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13. ABSTRACT (Maximum 200 Words)

Cancer growth and spread depends on the orchestrated proliferation of tumor-associated blood supply. Cancer cells release signals that instruct the body to build new blood vessels, angiogenesis, required to feed the tumor as it increases in size. Pharmacological agents, i.e. proteins and derivatives, that interfere with angiogenesis, in cancer bearing mice, stop cancer growth and lead to its regression. Animal modeling has revealed that repeated administration of large amounts of such antiangiogenic proteins is required for anti-cancer effect. This may be logistically difficult to achieve in larger beings such as humans. A remedy to this problem would involve a combined cell and gene therapy approach. We propose that normal tissue such as marrow stromal cells (MSCs) can be harvested from patients, genetically engineered, and subsequently returned to the patient as an implant releasing on a continuous basis therapeutic proteins that interfere with cancer growth and spread. We have already developed and published most of the key components required to develop this novel therapeutic modality, such as vectors, MSCs, and matrices. Also thus far, we have shown significant decrease in tumor progression over time with Interleukin-12-secreting MSCs in the 4T1 mouse model of breast cancer and ascertained reproducibility of these results.

14. SUBJECT TERMS
Autologous cells, marrow stroma, breast cancer, cell and gene therapy, angiogenesis

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Annual Summary Report for Postdoctoral Fellowship BC011316 "Genetically Engineered Autologous Cells for Antiangiogenic Therapy of Breast Cancer" DAMD 17-02-1-0477

KEY RESEARCH ACCOMPLISHMENTS

The major objectives of Task 1 of the Statement of Work have been completed, i.e.
Task 1: *Production and characterization of novel retroviral vectors comprising antiangiogenic genes, such as Interleukin-12 (IL-12), Months 1-16.*
Specifically, with regards to the aims of Task 1 of the Statement of Work shown below in italic font,

a. *Constructs*
The cDNA for murine IL-12 was kindly provided by Dr. Yukio Nakamura.

b. *Clone the cDNAs into our previously published retrovector constructs and generate replication-free retroparticles.*
We cloned the cDNA for IL-12 into a retrovector construct and thus generated the IL-12 retrovector, as well as IL-12 replication-free retroparticles. In addition, we similarly produced a Control retrovector not expressing IL-12, as well as replication-free Control retroparticles.

c. *Transduce primary marrow stromal cells (MSCs) and determine gene transfer efficiency, vector stability, transgene expression, and antiangiogenic protein secretion levels from polyclonal, as well as monoclonal populations.*
Using IL-12 retroparticles, we transduced primary MSCs from Balb/c mice, as well as from C57Bl/6 mice, and resulting polyclonal and monoclonal preparations of murine MSCs secreting IL-12 were assessed *in vitro*. We noted high gene-transfer efficiency and efficient transgene expression. Enzyme-linked immunosorbent assay (ELISA) revealed that polyclonal and monoclonal IL-12 MSCs populations secrete *in vitro* over 20ng IL-12/10^6 cells/24hrs. Furthermore, we likewise prepared Control MSCs which consisted of MSCs gene-modified with Control vector retroparticles and ascertained, by IL-12 specific ELISA conducted on supernatant, no IL-12 secretion by these Control cells, as expected.

Part of the objectives of Task 2 of the Statement of Work have been completed, i.e.
Task 2: *Determination of the therapeutic efficacy of transplanted gene-modified autologous MSCs in breast cancer-bearing mice. The tasks below will be accomplished with antiangiogenic gene product IL-12 with and without other antiangiogenic gene products, Months 17-36.*
Specifically, with regards to the aims of Task 2 of the Statement of Work shown below in italic font,

a. *Implant Balb/c mice (over 50 with controls) with non-metastatic DA3 murine mammary adenocarcinoma and later implant subdermally a removeable organoid comprising viscous collagen-embedded MSCs genetically engineered to secrete the antiangiogenic gene product(s).*
We have prepared the appropriate IL-12 Balb/c MSCs and Control Balb/c MSCs, have obtained the DA3 cells, and the viscous collagen matrix necessary for the above aim but have not yet conducted this particular implantation experiment. This will be carried out in the fall of 2004.

b. *Implant Balb/c mice (over 50 with controls) with metastatic 4T1 murine mammary adenocarcinoma (animal model of stage IV human breast cancer) and later implant subdermally a removeable organoid comprising viscous collagen-embedded MSCs genetically engineered to secrete the antiangiogenic gene product(s).*
We conducted this aim in its entirety as well as supplemental related experiments and have appended our results, represented as figures, to this renewal report. In brief, we show our findings utilizing MSCs embedded in two different collagen-based matrices, i.e. Matrigel™, which can be used in mice, and in Contigen™, which may also be employed in humans.

c. *Monitor tumor growth and correlate with transgene expression assessed by biochemical protein assay on plasma.*
We determined tumor growth and mouse survival over time for experimental animals implanted for aim b above. In certain cases, we executed ELISAs specific for IL-12 on plasma samples collected from mice.
As illustrated in the figures appended below, we demonstrated with 10^5 4T1 breast cancer cells a decrease in tumor progression when a polyclonal population of 5 x 10^5 IL-12-secreting Balb/c MSCs were implanted subcutaneously embedded in a collagen matrix in Balb/c mice. Control mice received tumor cells alone or with control vector modified MSCs. Before conducting more implantations with the Balb/c derived MSCs in the isogenic 4T1 breast cancer model, we wanted to first ascertain that the beneficial effect achieved with the IL-12 gene-modified Balb/c MSCs was not just specific to one mouse strain. Thus, using the IL-12 gene-modified C57Bl/6 MSCs in the isogenic B16 melanoma model, we showed with 10^5 B16 cells a decrease in tumor progression when a polyclonal population of 5 x 10^5 IL-12-secreting C57Bl/6 MSCs were implanted subcutaneously embedded in a collagen matrix in C57Bl/6 mice.
Since we were reassured that the effect was not mouse strain-specific, experiments that we subsequently carried out were focused on the 4T1 breast cancer model. We demonstrated a greater decrease in tumor progression through the utilization of a monoclonal population of IL-12 Balb/c MSCs at a greater ratio of MSCs to breast cancer cells, i.e. 2.5 x 10^4 4T1 breast cancer cells and a monoclonal population of 10^6 IL-12-secreting MSCs. As shown in the appended figures, most experiments were performed with MSCs embedded in the matrix Matrigel™ and some with MSCs embedded in the matrix Contigen™. Control mice received tumor cells alone or with control vector modified MSCs.
These results will be the focus of an abstract prepared for presentation at a scientific meeting in 2005 and of a manuscript in preparation.

d. *Likewise, implant the gene-modified MSCs subdermally in nude mice (over 50 with controls) bearing breast cancer xenografts (such as MDA235) and determine tumor response as well as extent and duration of transgene expression in MSCs by periodic peripheral blood sample analysis.*
We already have available the appropriate murine IL-12 MSCs and Control MSCs, as we have generated MSCs appropriate for different implantation experiments, but we have not yet executed this particular obective because we felt it important to first perform the following experiments for which results, represented as a figure, have also been appended to this renewal report.
In brief, we conducted experiments in immunodeficient mice, specifically NOD-SCID mice, which we implanted with 4T1 breast cancer cells and the following day with IL-

12-secreting Balb/c MSCs embedded in a collagen-based matrix and showed absence of a therapeutic effect and consequently the importance of the immune system to achieve a beneficial response in the host. This suggests that the direct anti-tumor effect of IL-12 on its own is insufficient. Alternatively, the anti-tumor effect of IL-12 may be mediated via bystander immune cells

e. *Perform immunohistochemical analysis on tumor sections examining vascular and related structures from all groups of mice.*
For several experiments carried out thus far, tumors and organs have been harvested from mice and sections prepared. These tissues will also be collected from mice in future planned experiments. We have already established that a veterinary pathologist will offer further expert analysis and interpretation of our here findings.

f. *Conduct mechanistic analysis of the antiangiogenic effect of IL-12. Specifically, (1) determine if host-derived vascular structures express the IL-12 receptor and reveal effect on cell biology and (2) identify host-derived immune competent cells recruited by IL-12 and analyze their role in the antiangiogenic effect.*
This aim has not yet been conducted. Some preliminary findings are however expected in Fall 2004.

Please note that experiments that have not yet been conducted are those we planned for the third year of the proposal.

REPORTABLE OUTCOMES

Research

Manuscripts published in 2003 and 2004

- Al-Khaldi, A., **Eliopoulos, N.**, Lejeune, L., Martineau, D., Lachapelle, K., Galipeau, J. Postnatal Bone Marrow Stromal Cells Elicit a Potent VEGF-Dependent Neo-Angiogenic Response. Gene Therapy, 10(8): 621-629, 2003.

- Annabi, B., Thibeault, S., Lee, Y.T., Bousquet-Gagnon, N., **Eliopoulos, N.**, Galipeau, J. Béliveau, R. Matrix Metalloproteinase Regulation of Sphingosine-1-Phosphate-Induced Angiogenic Properties of Bone Marrow Stromal Cells. Experimental Hematology, 31: 640-649, 2003.

- Annabi, B., Lee, Y.T., Turcotte, S., Naud, E., Desrosiers, R.R. **Eliopoulos, N.**, Galipeau, J., Béliveau, R. Hypoxia promotes murine bone marrow-derived stromal cell migration and tube formation. Stem Cells, 21: 337-347, 2003.

- **Eliopoulos, N.**, Al-Khaldi, A., Crosato, M., Lachapelle, K, Galipeau, J. A Neovascularized Organoid Derived from Retrovirally-Engineered Bone Marrow Stroma Leads to Prolonged *In Vivo* Systemic Delivery of Erythropoietin in Non-Myeloablated, Immunocompetent Mice. Gene Therapy, 10(6): 478-489, 2003.

- Annabi, B., Naud, E., Lee, Y.T., **Eliopoulos, N.**, Galipeau, J. Vascular progenitors derived from murine bone marrow stromal cells are regulated by fibroblast growth factor and are avidly recruited by vascularizing tumors. Journal of Cellular Biochemistry, 91:1146–1158, 2004.

- **Eliopoulos, N.**, Lejeune, L., Martineau, D., Galipeau, J. Human-compatible collagen matrix for prolonged and reversible systemic delivery of erythropoietin in mice from gene-modified marrow stromal cells. Molecular Therapy, 2004, *In Press.*

Abstracts presented in 2003 and 2004

- **Eliopoulos, N.**, Lejeune, L., Martineau, D., Galipeau, J. Genetically Engineered Autologous Marrow Stromal Cells Sequestered Within a Human-Compatible Bovine Collagen Matrix for Prolonged and Reversible In Vivo Systemic Delivery of Functional Erythropoietin in Mice. Presented at the American Society of Gene Therapy 6th Annual Meeting, June 4-7, 2003, in Washington, DC, and at the Canadian Institutes of Health Research (CIHR)/BioContact Meeting, October 1-3, 2003, in Quebec city, Quebec, where it was awarded **first prize**.

- Fontaine, F., Dunn, M., Hernandez, J., **Eliopoulos, N.**, Boucher, H., MacLeod, J.N., Galipeau, J., Martineau, D. Autologous Canine Bone Marrow Stromal Cells Retrovirally Engineered with the Canine EPO Gene and Implanted *In Vivo* in a Collagen Matrix Produce and Release Systemically Functional EPO over a Prolonged Period. Presented at the American Society of Gene Therapy's 7th Annual Meeting, June 2-6, 2004, Minneapolis.

- **Eliopoulos, N.**, Stagg, J., Lejeune, L., Galipeau, J. MHC Class I and II Mismatched Marrow Stromal Cells from C57Bl/6 Mice are Immune Rejected by Recipient Balb/c Mice. Presented at the American Society of Gene Therapy 7th Annual Meeting, June 2-6, 2004, Minneapolis.

Comments on manuscripts and abstracts listed above

The published (or accepted) peer-reviewed articles listed above show compelling data that strongly predict that a "transgenic cell therapy" approach with antiangiogenic gene product-secreting MSCs would be feasible for the treatment of breast cancer. These extensive "proof-of-principle" studies are of importance to analyze the utility of MSCs as a delivery vehicle for functional plasma soluble proteins, such as murine IL-12 in mice.

We have investigated the use of "matrix" embedded MSCs which are subsequently implanted in the subcutaneous space, the concept being that an artificial subcutaneous "organoid" would be formed from which the plasma protein would be produced. Thus, if any unpredictable undesirable side effects from the genetically engineered MSCs were to occur or if the effect is no longer needed, it would be possible, as we demonstrate in our latest manuscript, to remove the "implant". Also, we noted that this "delivery platform" leads to a more sustained and more significant effect in test mice, as compared to implantation of "free" non-embedded MSCs. Since for eventual clinical application, we desire a "human-compatible" matrix for immunological compatibility, we showed, as reported in our latter manuscript, the effective utilization of a FDA-approved, bovine collagen-based viscous matrix to support MSCs for cell and gene therapy applications.

Moreover, due to indications in the literature that MSCs may serve as "universal donors", we conducted a study to determine if the implantation of primary murine MSCs genetically engineered to release a soluble protein would be feasible in MHC-mismatched allogeneic

mice without immunosuppression. Our findings revealed that class I and II MHC-mismatched MSCs will elicit a cellular immune response by allogeneic hosts with normal immune systems, and strongly suggest that MSCs, at least in the murine system, cannot serve as a "universal donor" in cell and gene therapy applications, important findings if one is to contemplate translation of this approach to humans.
In sum, our overall results robustly suggest that matrix implants comprising gene-modified MSCs can be effectively utilized as platforms for the systemic delivery of plasma soluble therapeutic proteins, such as IL-12, and that the facility of their implantation and possible retrieval renders this approach clinically desirable and feasible.

FURTHER TRAINING and AWARD

Excellent mentoring has been imparted to me by my supervisor, Dr. Jacques Galipeau, for experimental work but also for the eventual transition from a senior postdoctoral fellow to a junior scientist. Accordingly, I have been increasingly implicated in grant proposal writing, in offering experimental advice to graduate students and summer students, and in collaborating with colleagues that has led to co-authorships on abstracts and manuscripts.

In addition, training has also been provided by regular lab meetings and discussions with supervisor, colleagues and collaborators, by following lectures by invited speakers, as well as by attending and presenting at National and International scientific meetings.

Further very relevant knowledge to aid in the effective execution of the granted proposal was achieved by attending a course entitled "Critical Issues in Tumor Microcirculation, Angiogenesis and Metastasis: Biological Significance and Clinical Relevance" offered by Harvard Medical School, Department of Continuing Education, and Massachusetts General Hospital, in June 2004, in Cambridge, MA.

In addition, I was one of twelve participants selected across Canada to present an abstract at the Canadian Institutes of Health Research/BioContact Meeting in October 2003, in Quebec city, QC, where I was very honoured to have been chosen by a panel of judges to receive the first prize.

LIST OF APPENDED DOCUMENTS

1) Figures of results thus far obtained and directly related to the fellowship proposal. These will be the focus of an abstract submitted for presentation at a scientific meeting in 2005 and of a manuscript in preparation.
2) Manuscript entitled "Human-Compatible Collagen Matrix for Prolonged and Reversible Systemic Delivery of Erythropoietin in Mice from Gene-Modified Marrow Stromal Cells" by **Eliopoulos, N.**, Lejeune, L., Martineau, D., and Galipeau, J. Accepted in July 2004 for Publication in *Molecular Therapy.*
3) Abstract entitled "MHC Class I and II Mismatched Marrow Stromal Cells from C57Bl/6 Mice are Immune Rejected by Recipient Balb/c Mice" by **Eliopoulos, N.**, Stagg, J., Lejeune, L., and Galipeau, J. Presented at the American Society of Gene Therapy 7th Annual Meeting, June 2-6, 2004, Minneapolis, and Figures for a manuscript in preparation.
4) Certificate obtained through participation in the course entitled "Critical Issues in Tumor Microcirculation, Angiogenesis and Metastasis: Biological Significance and Clinical Relevance" offered by Harvard Medical School, Department of Continuing Education, and Massachusetts General Hospital, in June 2004, in Cambridge, MA.

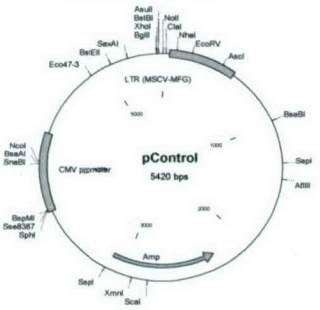
APPENDIX 1

Figures of results thus far obtained and directly related to the fellowship proposal. These will be the focus of an abstract submitted for presentation at a scientific meeting in 2005 and of a manuscript in preparation.

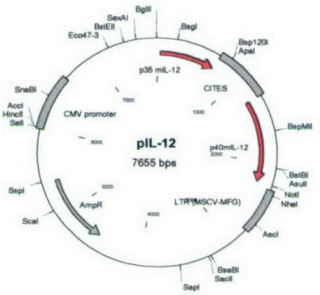
Main Results Thus Far Obtained for Funded Postdoctoral Fellowship Proposal

Retroviral Constructs:

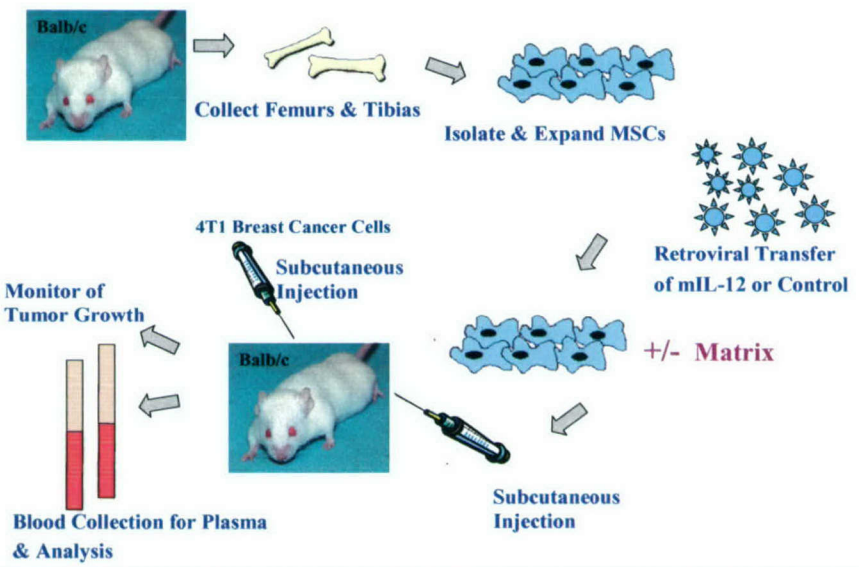
Control Vector



mIL-12 Vector



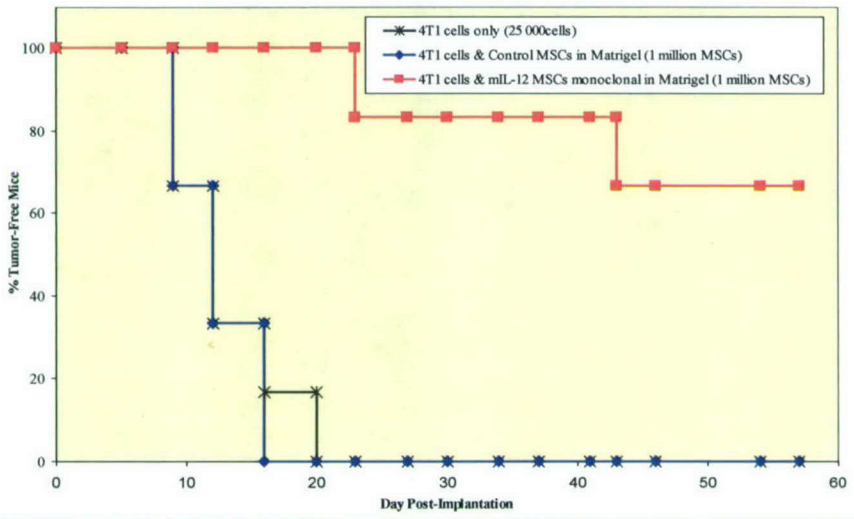
Experimental Outline



Results of *in vivo* experiments in Balb/c mice implanted with **2.5 x 10^4** 4T1 cells & 24 hrs later with **10^6** gene-modified Balb/c MSCs monoclonal population in matrix **Matrigel.** Experiment was conducted 4 times with n=5-9 for each group of mice.

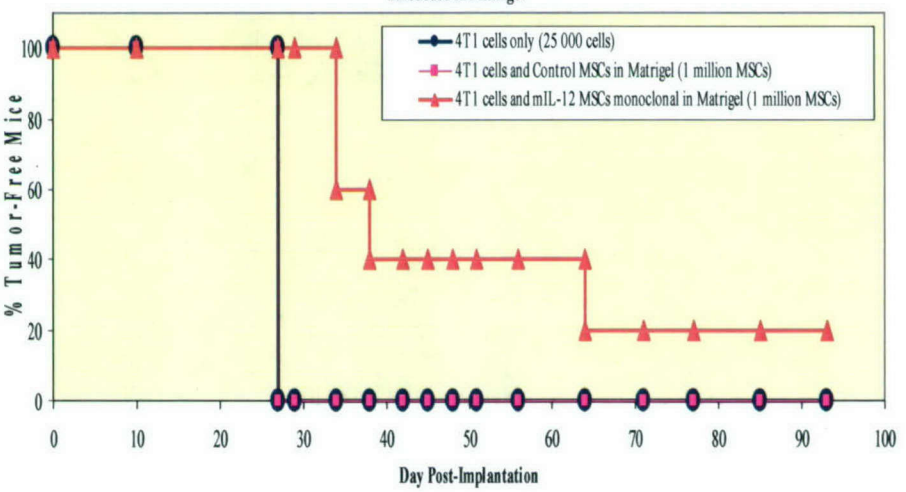
Experiment 1

Percentage of Tumor-Free Mice Following Implantation with 4T1 Breast Cancer Cells With/Without Gene-Modified MSCs Embedded in Matrigel

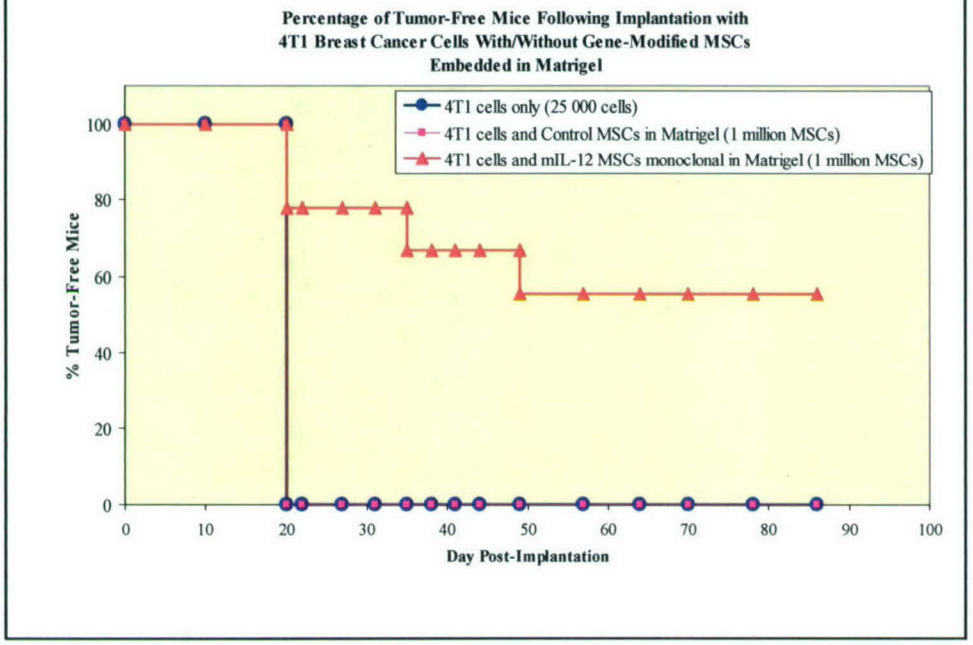


Experiment 2

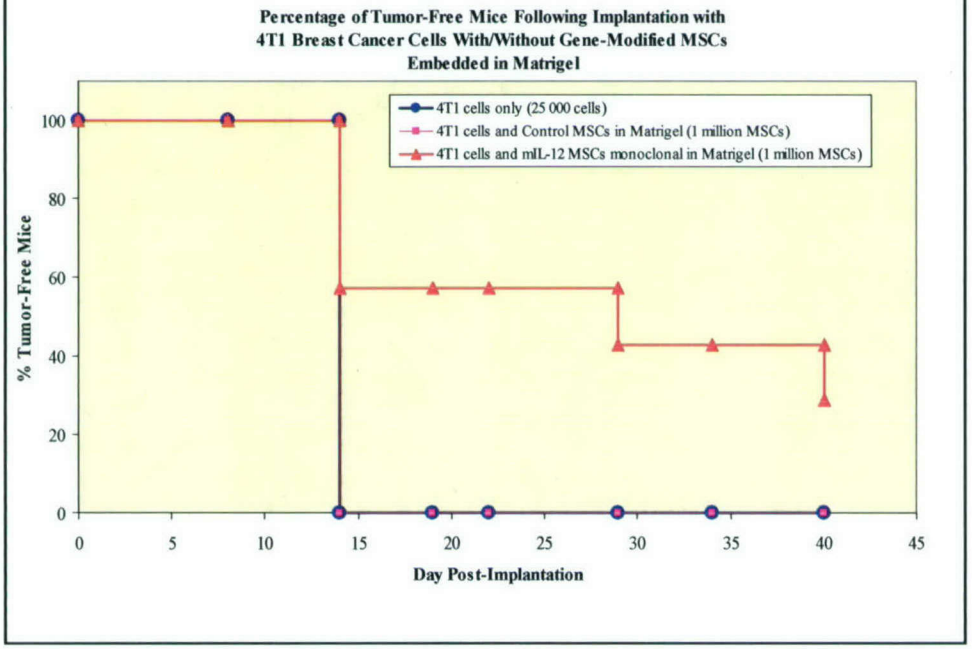
Percentage of Tumor-Free Mice Following Implantation with 4T1 Breast Cancer Cells With/Without Gene-Modified MSCs Embedded in Matrigel



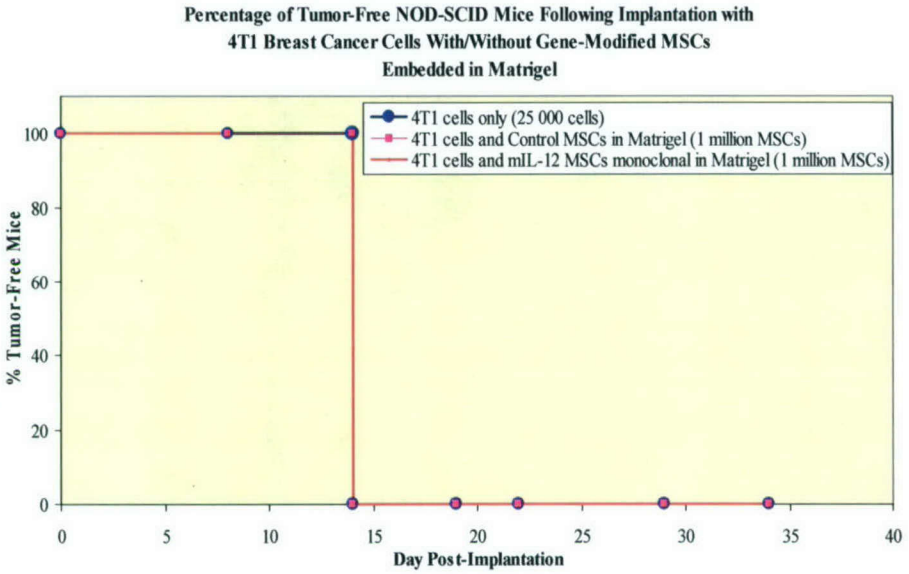
Experiment 3



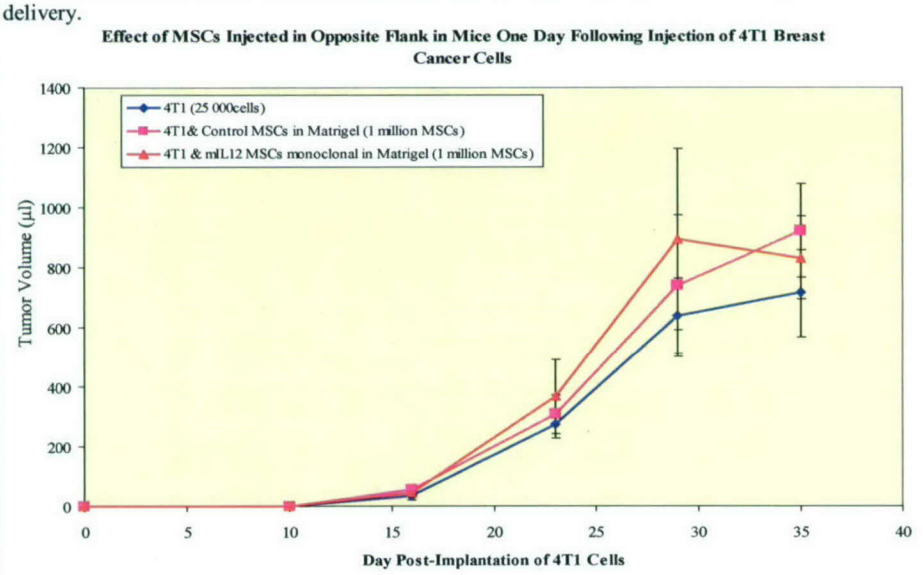
Experiment 4



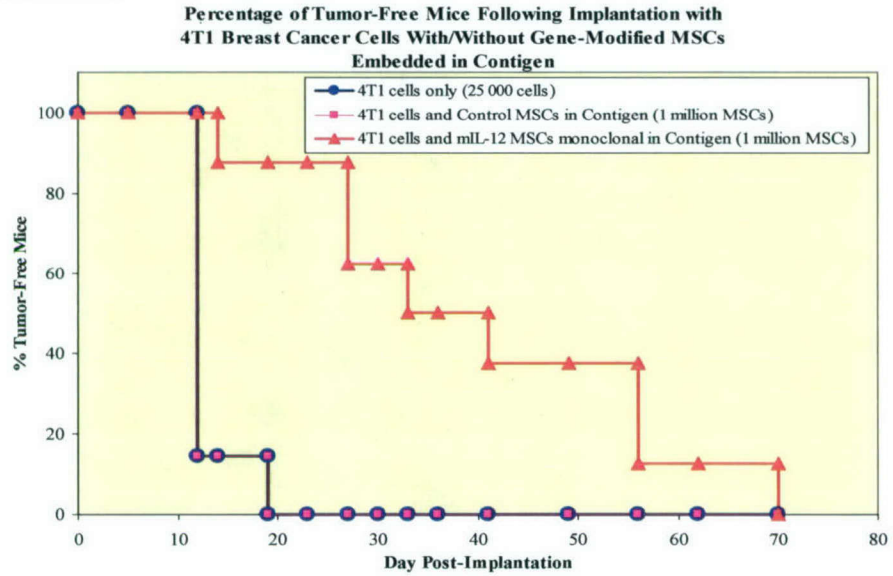
Results of *in vivo* experiment in immunodeficient (NOD-SCID) mice implanted with 2.5 x 10^4 4T1 cells & 24 hrs later with 10^6 gene-modified Balb/c MSCs monoclonal in matrix Matrigel (n=5-9), which determined that the effect with mIL-12 MSCs is immune mediated.



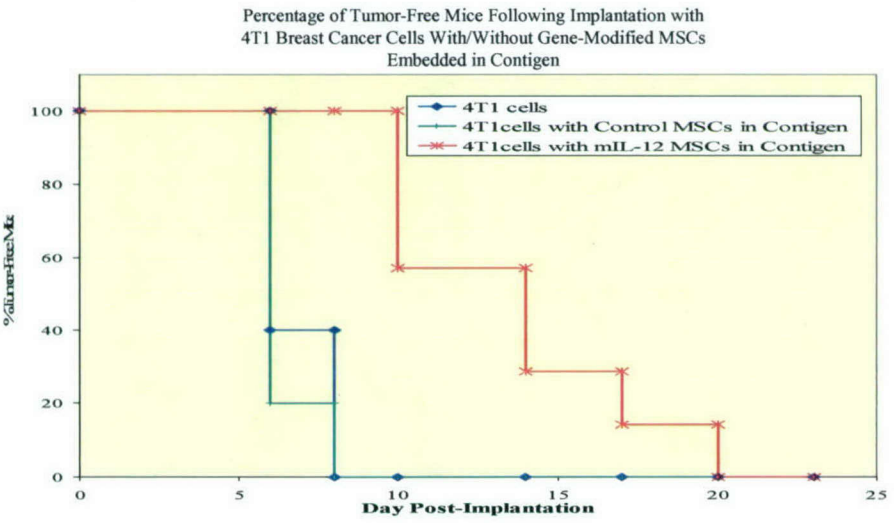
Results of *in vivo* experiment in Balb/c mice implanted with 2.5 x 10^4 4T1 cells & 24 hrs later with 10^6 gene-modified Balb/c MSCs monoclonal in matrix Matrigel (n=5-7) in the contralateral flank, which determined that the effect with mIL-12 MSCs is not due to its systemic delivery but to its local delivery.



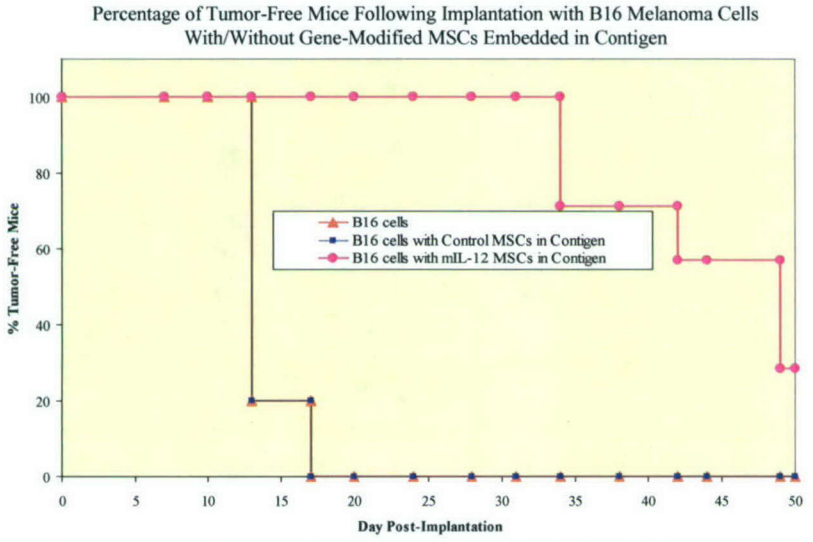
Results of *in vivo* experiment in Balb/c mice implanted with **$2.5x10^4$** 4T1 cells & 24 hrs later with **10^6** gene-modified Balb/c MSCs in matrix **Contigen** (n=5-8), which determined that mIL-12 MSCs embedded in a matrix other than Matrigel and that is human-compatible can lead to an effect in 4T1 breast cancer.



Results of *in vivo* experiment in Balb/c mice implanted with **10^5** 4T1 cells & 24 hrs later with **$5x10^5$** gene-modified Balb/c MSCs in matrix **Contigen** (n=5-8), which determined that mIL-12 MSCs embedded in a matrix other than Matrigel and that is human-compatible can lead to an effect in 4T1 breast cancer (Note: Tumor cells at a higher number and a polyclonal population of mIL-12 MSCs at a lower number).



Results of *in vivo* experiment in C57Bl/6 mice implanted with **10^5** B16 cells & 24 hrs later with **$5x10^5$** gene-modified C57Bl/6 MSCs in matrix **Contigen** (n=5-8), which determined that mIL-12 MSCs embedded in a matrix other than Matrigel and that is human-compatible can lead to an effect that is not strain specific and not only in 4T1 breast cancer but also in B16 melanoma (Note: Tumor cells at a higher number and a polyclonal population of mIL-12 MSCs at a lower number).



APPENDIX 2

Manuscript entitled "Human-Compatible Collagen Matrix for Prolonged and Reversible Systemic Delivery of Erythropoietin in Mice from Gene-Modified Marrow Stromal Cells" by **Eliopoulos, N.**, Lejeune, L., Martineau, D., and Galipeau, J. Accepted in July 2004 for Publication in *Molecular Therapy.*

Jacques Galipeau@JGH

05/07/2004 08:23 AM

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cc:
Subject: MTHE-D-04-00672R2 Accept

Congrats!

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04/07/2004 03:48 PM

To: <jacques.galipeau@mcgill.ca>, <jgalipea@lab.jgh.mcgill.ca>
cc:
Subject: MTHE-D-04-00672R2 Accept

Dear Dr. Galipeau:

Thank you for allowing me to review your manuscript, "Human-compatible collagen matrix for prolonged and reversible systemic delivery of erythropoietin in mice from gene-modified marrow stromal cells."

I am pleased to inform you that your manuscript has been accepted for publication in Molecular Therapy. Your production editor is Susan Ikeda (s.ikeda@elsevier.com) whom you should contact if you have not received proofs within about three weeks. Please note that we only accept figures for production that are in tif or eps format.

We would also like to take this opportunity to remind you that there is a charge of $50 per page for articles appearing in Molecular Therapy. These funds are collected by the American Society of Gene Therapy and are used to improve the journal's coverage of news and issues affecting the gene therapy community.

With many thanks for submitting your paper to Molecular Therapy. I look forward to seeing it in print!

Best regards,

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Human-compatible collagen matrix for prolonged and reversible systemic delivery of erythropoietin in mice from gene-modified marrow stromal cells

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ABSTRACT

Bone marrow stromal cells (MSCs) can be exploited therapeutically in transgenic cell therapy approaches. Our aim was to determine if gene-modified MSCs sequestered within a clinically approved, bovine type I collagen-based viscous bulking material could serve as a retrievable implant for systemic delivery of erythropoietin (Epo). To test this hypothesis, we embedded Epo-secreting MSCs in viscous collagen (Contigen™) and determined the pharmacological effect following implantation in normal mice. Primary MSCs from C57Bl/6 mice were retrovirally-engineered to express murine Epo (mEpo) and 10^7 cells of a clonal population secreting 3U of mEpo/10^6cells/24hrs were implanted subcutaneously in normal C57Bl/6 mice with and without viscous collagen. Without matrix support, Hct rose to >70% for <25 days and returned to baseline by 60 days. However, in mice implanted with viscous collagen-embedded MSCs, the Hct rose to >70% up to 203 days post-implantation ($p<0.0001$). In parallel, plasma Epo concentration was significantly increased ($p<0.05$) for >145 days. Moreover, surgical removal of the viscous collagen organoid 24 days ensuing implantation led to reduction of Hct to baseline levels within 14 days. In conclusion, this investigation demonstrates that mEpo$^+$MSCs embedded in human-compatible viscous collagen matrix offers a potent, durable and reversible approach for delivery of plasma soluble therapeutic proteins.

Keywords

marrow stroma, collagen, erythropoietin, cell and gene therapy, autologous cells

INTRODUCTION

Bone marrow derived stromal cells (MSCs) are an autologous cell type that can be made use of for numerous cell and gene therapy applications [1-4]. We have previously reported [5] the pharmacological effect on blood hematocrit of a synthetic endocrine organoid derived from erythropoietin (Epo)-transduced MSCs admixed in a basement membrane gel Matrigel™ (Becton Dickinson Biosciences, MA). However, Matrigel is derived from a murine sarcoma cell line, thus non-human compatible, which prohibits its use in clinical studies. Since collagen is an important component of Matrigel, we also evaluated a clinically-applicable bovine type I collagen porous formulation, namely Collagen Matrix (Collagen Matrix Inc., N.J.), as a support vehicle for Epo-releasing MSCs. The observed results supported the notion that a bovine collagen-based matrix could serve as a viable platform to support MSCs *in vivo*, though there was much room for formulation improvement. Indeed, there is extensive published experience in testing a wide array of biological and synthetic matrices to support engineered somatic cells *in vivo* [6-10]. However, we sought a formulation that offered cost-effective, FDA-approved, off-the-shelf convenience, coupled to specific pharmacological properties and allowing for removal of the organoid when the pharmacological effect is no longer required or if unforeseen side effects were to arise from the embedded gene-modified cells.

Contigen™ (C.R. Bard Inc., GA) is a clinically approved, bovine collagen-based formulation, utilized as a peri-urethral injectable viscous bulking agent for the treatment of stress urinary incontinence in women [11,12]. Specifically, it is a nonpyrogenic substance constituted of highly purified dermal collagen, 95% Type I with ≤5% Type III, suspended in phosphate buffered physiological saline (35mg/ml). Unlike many clinical-grade collagen formulations, it has a pH of 7.3 and is designed for a long half-life *in vivo*. We therefore tested the utility of this viscous collagen preparation as the matrix component of a human-compatible synthetic endocrine organoid. We here report, as a proof of concept, that in mice injected subcutaneously with viscous collagen-embedded Epo-secreting MSCs, a pharmacological effect arose that was superior to and of significantly longer duration than that seen with MSCs without matrix support. Furthermore, we ascertained the flexibility of the approach by reversing the clinical effect via surgical removal of the organoid implant. These results buttress the use of bovine collagen-based matrices for pre-clinical and clinical studies incorporating autologous, engineered somatic cells – such as MSCs – as a biopharmaceutical platform for long term delivery of plasma soluble therapeutic proteins.

RESULTS

Epo Gene-Modified Marrow Stroma

To confirm that mEpo transduced MSCs secrete mEpo *in vitro*, and quantitate the amount, supernatant collected from these MSCs was utilized in an ELISA for human Epo as previously described [5]. The polyclonal population of Epo^+MSCs was determined to secrete ~2 U of Epo per 10^6 cells per 24 hours and the clonal population of Epo^+MSCs, utilized in the present study, was noted to release ~3 U of Epo per 10^6 cells per 24 hours. There was no Epo detected in the supernatant collected from control MSCs (data not shown). Flow cytometric analysis of the clonal subset of Epo^+MSCs utilized experimentally revealed a phenotype of <0.01% CD31+, 98% CD44+, <0.01% CD45+ and 35% CD34+.

Long-Term Hematocrit of Mice Implanted Subcutaneously with Epo-Secreting MSCs

We assessed and compared the long-term effect on hematocrit (Hct) of MSCs delivered subcutaneously, either admixed in viscous collagen or without matrix support. As observed in Figure 1, in C57Bl/6 mice (n=5) implanted with 10^7 collagen-embedded Epo^+MSCs, the hematocrit rose from a basal 51 ± 0.2% (Mean ± SEM) pre-implantation to 81 ± 0.9% at 22 days following implantation and remained at values of 82-88% until day 106, and surpassing 70% up to day 203. Control mice implanted with collagen only (Figure 1) or with IRES-GFP engineered MSCs in collagen (data not shown) showed stable baseline Hct levels over time. In contrast, when the identical amount of 10^7 Epo^+MSCs were injected subcutaneously without matrix support, the Hct increased from a basal 57 ± 0.7% prior to implantation to a peak value of 70 ± 3.2% at 23 days post-implantation which gradually thereafter decreased, reaching basal value of 57 ± 2.4% at ~63 days (Figure 1). When comparing the long-term impact on Hct, all mice implanted with collagen embedded Epo^+MSCs sustained a Hct of ≥70% for over 119 days whereas in mice which received unembedded cells, this Hct level persisted for 23 days in 4 of 5 mice (p=0.0001 LogRank). We observed that the decrease in Hct in the matrix group was associated with the physical decrease in size of the implant, likely due to gradual resorption.

Plasma Epo Concentration in Mice Implanted with Epo-Secreting MSCs in Contigen

We measured the concentration of mouse Epo in plasma of mice over time with the use of a human Epo ELISA assay. In mice injected with collagen embedded Epo^+MSCs, plasma Epo levels rose from a basal 7.5 ± 0.5 mU/ml before implantation to 25-65 mU/ml, as early as 6 days post-implantation (33 ± 2.6 mU/ml), peaking at 14 days (67 ± 17 mU/ml) and slowly falling to levels of 25 ± 7.2 mU/ml at day 93 (Figure 2). Thereafter, the concentration of Epo in plasma further decreased to 15 ± 3.0 mU/ml at day 147. Statistical evaluation of the plasma Epo concentrations in recipient rodents of Epo^+MSCs embedded in collagen revealed that values detected at days 6 to 147 were significantly different ($P<0.05$, Student t-test) from pre-experiment baseline measurements (Figure 2). Moreover, although a peak in plasma Epo concentration was observed at day 14, statistical analysis revealed that values measured at days 6 to 93 inclusively were not significantly distinct from one another. It was only from time point day 119 that the concentration of Epo in plasma was significantly different from that measured at day 14 ($P<0.01$, Student t-test). Further, from day 163 on ensuing implantation, plasma Epo levels detected were not significantly dissimilar from baseline. An ELISA assay specific for detection of human Epo was used to measure mouse Epo as is standard and similarly utilized in other studies [5,13,14]. Hence, the sensitivity for mEpo being weak [15], our measured plasma mEpo levels are likely underestimated but remain useful for comparison with other published reports. Epo ELISA was similarly conducted on

plasma from mice implanted with Contigen only or with Contigen-embedded IRES-GFP enegineered MSCs and values were below 10mU/ml (data not shown).

Removal of Contigen-Embedded MSCs and Abolishment of Pharmacological Effect.
The organoid implant behaves as a synthetic endocrine gland. Its removal should lead to complete abolishment of its pharmacological effects if the bulk of engineered MSCs remain within its framework. To test this hypothesis, nine mice were implanted subcutaneously with Epo^{+}MSCs embedded in collagen and implants were removed from 4 randomly chosen mice 24 days later. As illustrated in Figure 3, implants were easily harvested from live mice under anesthesia with no residual matrix remaining post-surgery and no morbidity. The Hct in these 4 mice decreased from 77 ± 2.7% at 21 days post-implantation to baseline levels of 55 ± 1.2% within 14 days following implant removal, whereas in mice with implant left intact, the Hcts remained significantly increased at >75% for the duration of the experiment ($P \leq 0.005$, Student t-test) (Figure 4).

Histological Analysis of Contigen Implants Following Removal from Mice

Organoids were removed from mice implanted 24 days earlier with (i) collagen matrix only, (ii) EmptyVector engineered MSCs embedded in collagen matrix, in addition to (iii) Epo^{+}MSCs admixed in collagen matrix. The macroscopic appearance of the implants was similar in the three groups. Microscopically, the implants consisted of a large, fragmented avascular center surrounded by a thin band of vascularized matrix material, itself covered by a capsule of connective tissue made of mature collagen infiltrated by scant neutrophils and richly vascularized by a loose network of capillaries (Fig. 6). The capillary network extended into a thin subjacent band of matrix material that contained viable cells. In the implants containing MSCs, the fragments composing the center contained dead cells that showed features of coagulation necrosis. The minimal inflammatory response consisted of neutrophils scattered within the capsule and the neovascularized matrix band. The adjacent subcutaneous layer contained several dense perivascular groups of plasma cells filled with Russell bodies. There were no obvious differences between the three groups examined, and thus the host-derived response was due to the collagen material and not the MSCs. The collagen matrix material is quite inert as it triggers little inflammation.

Analysis of MSC Phenotype Prior to and Post Implantation in Mice within Contigen Matrix

The phenotype of polyclonal GFP-labelled MSCs (GFP^{+}MSCs) prior to implantation was <0.04% CD31+, 96% CD44+, <0.1% CD45+ and 15% CD34+ (Figure 6A). GFP reporter expression allows the use of cell sorting to distinguish our GFP^{+}MSCs from host-derived infiltrating cells. Flow cytometry analysis was performed on GFP^{+}MSCs retrieved from implants 23 days following subcutaneous injection in mice and showed the same phenotype for expression of CD44 and CD45 as that observed prior to *in vivo* implantation (Figure 6B). Too few cells were recovered from implants for accurate analysis of CD31 and CD34 levels.

DISCUSSION

Collagen constitutes a valuable biomaterial for medical purposes [16]. In this study, we established that a three-dimensional, clinically-approved bovine type I collagen-based viscous bulking preparation can serve as an effective matrix biomaterial for support of MSC-derived synthetic endocrine organoid. The long-term pharmacological effect observed was comparable to what we have previously noted using the mouse-specific Matrigel™ matrix [5].

Numerous studies have assessed three-dimensional matrices in pre-clinical models of transgenic cell therapy with various cellular vehicles including MSCs, myoblasts, and in many cases, fibroblasts. The type of materials tested fall under two broad categories: biomaterials and synthetic matrices and devices. Both types have been coupled to MSCs engineered to produce plasma soluble proteins, including: hydroxyapatite particles, hyaluronic acid sponge, and collagen-based sponge [8,9]. Indeed, others and we have previously validated [5,8,9] the utility of embedding protein-secreting MSCs within a collagen-containing matrix for prolonged pharmaceutical effect. As demonstrated by Daga et al. [8] with Epo-transduced human MSCs tested *in vivo* in 3 dissimilar matrices, including a collagen sponge, the pharmacological effect was of longer duration than without matrix support. The apex in the Hct upsurge with their collagen sponge device was ~60% at day 28 which remained at levels above 55% to day ~50 ensuing implantation. Another recent investigation led to human plasma factor IX serum levels, above 25, 11.5, and 6ng/ml, for 1 week, 1 month, and 4 months, respectively, in immunocompromised mice by MSCs enclosed in a collagen sponge [9]. Unlike the viscous collagen material here tested, the collagen materials brought into play in these two studies required a surgical procedure for subcutaneous implantation and led to short lived, modest or subtherapeutic plasma protein levels. Thus, the "injectability" of a viscous collagen matrix coupled to robust long-term support of engineered MSCs as we have here shown stand out as desirable features.

Similarly, mammalian adult somatic cells other than MSCs, such as fibroblasts and myoblasts engineered to produce plasma soluble proteins, have been embedded in an array of collagen-based biomaterials [7,17-20]. Though conceptually related, most of these biomatrices have not been validated for use in humans, and serve mostly as buttressing proof-of-concept experiments validating in a generic sense the use of collagen-based matrix materials.

Synthetic matrices and surgically implanted devices have also been widely tested [6,10,14,21-27], including 5% agarose gel, microcapsules, and immunoisolatory devices. An obvious advantage of these strategies is the avoidance of all biological materials in an implant device. However, most have remained at the pre-clinical prototype developmental stage and are still far removed from clinical approval and widespread clinical use.

Others, and we have also shown the use of genetically engineered MSCs for cell and gene therapy applications via intraperitoneal or intravenous administration [5,28,29]. The main downside to this mode of cell delivery is the inability to remove or retrieve the engineered cells when the pharmacological effect is no longer required or if an unforeseen toxicity were to arise from their use. However, a subcutaneous implantation would be desirable since MSCs confined to a three-dimensional scaffolded organoid would allow for surgical extraction if clinically warranted. Indeed, we unambiguously demonstrate that a collagen-based organoid is readily resectable and with it removal leads to the complete

reversal of its endocrine effect. This observation also leads us to speculate that the bulk of engineered MSCs sequestered within the implant do not migrate out of the organoid, at least in the first few weeks.

We also noted that although Contigen™ is human-compatible, it was not entirely inert in mice as a modest inflammatory infiltrate was observed following implantation. As illustrated in Figure 5, neutrophils and plasma cells infiltrated the matrix implant capsule. This host-derived inflammatory response was due to the bovine collagen material and not the MSCs as similar observations were made with the collagen matrix in the absence of MSCs. Moreover, a greater cell density was observed in the collagen implant periphery while a sparse and more necrotic cell population was seen in the implant core (Figure 5). There was little neovascularization, as if the collagen material was a barrier for new capillaries. It is possible that the glutaraldehyde cross-linking of this material contributes to prevent its neovascularization. This failure to vascularize most likely contributed to the death (coagulation necrosis) of implanted MSCs localized in the implant core and to the fragmentation of the organoid material.

We have noted from earlier studies with MSCs embedded in a matrix [5,30], that the therapeutic protein secreted by MSCs may for the most part diffuse through the matrix but that a small part can be directly released into the bloodstream as supported by our observation that some MSCs merge with blood vessels. This latter direct secretion into the systemic circulation would thus constitute the only effective means of plasma soluble gene product delivery for proteins where diffusion rates through the matrix are limiting. Marrow stromal cells have remarkable cellular plasticity and we have previously shown that a significant subset of these will adopt spontaneously an endothelial phenotype *in vivo* when embedded in Matrigel [30]. Interestingly, we saw no obvious phenotype change in MSCs embedded in Contigen *in vivo*, at least in the first three weeks following implantation, utilizing the GFP reporter labeled MSCs. As reported by others in the field, we noted anchorage-dependent MSCs to be CD31-, CD44+, CD45- *in vitro* [3] prior to implantation and remained as such following retrieval from Contigen. We speculate that though collagen-based matrix allows for survival of MSCs, the viscous collagen preparation here used lacks supplementary signals that would otherwise lead to some vasculogenic differentiation as we have previously observed in Matrigel. Though we did not observe any vasculogenic differentiation of MSCs, this did not seem to impede the desirable biopharmaceutical features of the collagen-based organoid in regards to its ability to support long-term, sustained delivery of proteins *in vivo*.

We chose to focus on a market-approved collagen device for a series of reasons. Namely, it is a pH neutral, viscous, collagen-based preparation specifically designed for delivery by injection in subcutaneous soft tissues and for slow resorption rate in humans. Though we presume its original clinical development had absolutely nothing to do with cell therapy, the pharmacological features it possesses are ideal for the purpose of providing a stable extra-cellular matrix environment for engineered autologous adult somatic cells as here described. Furthermore, its widespread clinical use makes it – and comparable devices - conveniently available for off-label use – in appropriate experimental setting – for cell therapy applications in the treatment of disease amenable to delivery of plasma soluble proteins in mammals, including humans. It is conceivable that the approach of plasma soluble protein delivery by viscous collagen encapsulated MSCs can be translated to numerous clinical purposes where a short-term or long-term beneficial effect is needed. Thus, diseases such as cancer, hemophilia, growth or other hormone deficiency, and all diseases

amenable to therapeutic plasma protein delivery could be improved via this neo-organoid therapeutic platform.

Our previous work [5] where we embedded Epo producing murine MSCs in Matrigel and implanted these in mice with intact immune systems detailed a proof-of-concept. Matrigel however, with its complex murine proteinaceous components, is compatible immunologically with mice only. If we were to extend our therapeutic platform to humans with normal immune systems, we could not use Matrigel due to anticipated immunological incompatibility and rejection of the implant. The work described in our present study reliably reproduces our results observed with Matrigel with an alternate "off the shelf" matrix material derived from bovine collagen – Contigen – which is already approved by the FDA for use in humans. Hence, we demonstrate that this technological platform can be clinically translated to use in humans. The supplementary benefit that this implant is removable and its pharmacological effect reversible, adds to the practical and ethical use of genetically engineered cells in otherwise normal humans and minimizes risk – albeit small – of these technologies.

Although it is assumed that engineered MSCs from large mammals – such as humans – will behave similarly *in vivo* to that observed in a murine model, the assumption must be tested and the use of MSCs as a platform to deliver therapeutic plasma proteins – such as Epo – needs to be validated independently in a large mammal animal model. Therefore, to address any potential unexpected problems, we have initiated a phase I clinical study assessing our approach combining Epo-engineered autologous MSCs and Contigen matrix in normal outbred beagles. Their immunological, physiological and genetic similarities to humans [33,34] makes this pre-clinical testing a robust means of validating engineered autologous MSCs as a practical and useful means of delivering therapeutic plasma proteins prior to human clinical trials.

MATERIALS AND METHODS

Production of Retrovector and of Retrovirus-Producing Cells

The retroviral plasmid pIRES-EGFP was formerly synthesized in our laboratory [31]. The retroviral construct pEmptyVector was generated by removal of IRES-EGFP fragment following NotI digest of pIRES-EGFP and subsequent autoligation of the resulting vector EmptyVector. The retrovector pEpo was constructed by retrieving the mouse Epo cDNA by BamHI digest of our previously reported pEpo-IRES-EGFP [5] and ligating it with a BglII digest of pEmptyVector.

For the preparation of retrovirus-producing cells GP+E86-Epo, the pEpo construct (10μg) was linearized by FspI digest and co-transfected, using lipofectamine reagent (Invitrogen/Life Technologies, Carlsbad, CA), with 1μg pEGFPC1 (Clontech), which contains the neomycin resistance gene, into the GP+E86 ecotropic retrovirus-packaging cell line [32] from American Type Culture Collection (ATCC). These cells were grown in Dulbecco's modified essential medium (DMEM) (Wisent Technologies, St.Bruno, QC) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Wisent) and 50 U/ml penicillin, 50μg/ml streptomycin (Pen/Step) (Wisent). Stable transfectants were selected in complete media supplemented with 400μg/ml geneticin (Invitrogen/Life Technologies). Control GP+E86-EmptyVector virus producers were generated in an identical manner and then sorted based on GFP expression, and GP+E86-IRES-EGFP cells were prepared and sorted as earlier published [5].

Collection, Culture, and Transduction of Primary Murine Marrow Stroma

One female 15-20g C57Bl/6 mouse (Charles River, Laprairie Co., QC) was sacrificed and whole marrow obtained by flushing the femurs and tibias with complete media (DMEM supplemented with 10% FBS and 50U/ml Pen/Strep). Cells were plated in tissue culture dishes and ensuing a five day incubation at 37°C with 5% CO_2, the non-adherent hematopoietic cells were discarded and the adherent marrow stromal cells (MSCs) cultured for ~15 passages in complete media. A mixed population of Epo gene-modified MSCs was generated by several transduction rounds of MSCs with retroparticles from subconfluent GP+E86-Epo producers. Specifically, transduction was carried out twice a day for three consecutive days in each of four successive weeks by placing 0.45μm filtered retroviral supernatant from virus producers over ~60% confluent MSCs, in the presence of 6μg/ml lipofectamine reagent (Invitrogen/Life Technologies). The monoclonal population of Epo-MSCs was generated by plating the polyclonal population at limiting dilutions and subsequently selecting and expanding individual clones. Supernatant was harvested from polyclonal and monoclonal gene-modified MSCs and mouse Epo secretion was evaluated by ELISA specific for human Epo (Roche Diagnostics). A clonal Epo^{+}MSC subpopulation secreting 3U Epo/million cells/24 hrs was used for all subsequent experiments. Cells had undergone about 12-15 passages at time of use for implantation experiments. Control EmptyVector engineered MSCs were prepared as described above and IRES-EGFP MSCs were generated as earlier reported [5]. Animals were handled under the guidelines promulgated by the Canadian Council on Animal Care and with the Animal Welfare Act Regulations and other Federal statutes relating to animals and experiments involving animals, and adheres to the principles set forth in the Guide for Care and Use of Laboratory Animals, U.S. National Research Council, 1996.

Marrow Stroma Phenotypic Analysis

Epo^+MSCs were analyzed prior to implantation for expression of cell surface antigens. Cells were incubated with the following mAbs after Fc receptor blocking: PE-labeled rat anti-mouse CD45 (clone 30-F11), CD44 (clone IM7), biotin-conjugated rat anti-mouse CD31 (clone 30-F11), CD34 (clone RAM 34), isotypic controls PE labeled rat IgG2a, IgG2b and biotin-conjugated rat IgG2a (All from BD Pharmingen, San Diego, CA). Biotinylated Abs were revealed by TC-streptavidin (Caltag Laboratories, Burlingame, CA, USA). All cells were washed and acquired using a FACS Calibur flow cytometer (BD Immunocytometry systems) and analyzed with Cellquest software.

Marrow Stroma Implantation for Long-Term Blood Sample Analysis

For the implantation of "matrix-free" cells, Epo^+MSCs were trypsinized, concentrated by centrifugation, and 10^7 cells in 500µl of serum-free RPMI media (Wisent) were injected subcutaneously in the right flank of 5 syngeneic C57Bl/6 mice. For the subcutaneous implantations of matrix-embedded MSCs, 10^7 Epo^+MSCs were resuspended in 50µl of RPMI media, mixed with ~500µl of a "human-compatible", FDA approved bovine type I collagen-based material Contigen™ (C.R. Bard Inc, Covington, GA) and implanted by subcutaneous injection in the right flank of 5 syngeneic C57Bl/6 mice. Control mice were implanted with Contigen only or 10^7 IRES-EGFP MSCs mixed in Contigen (n=3 per group). Blood samples were collected from the saphenous vein of recipient mice with heparinized micro-hematocrit tubes (Fisher Scientific, Pittsburgh, PA) prior to and every ~1 or more weeks post-implantation and utilized to assess hematocrit (Hct) levels by standard microhematocrit method and plasma mEpo concentrations by ELISA for human Epo (Roche Diagnostics). Mice were followed for over 250 days.

Marrow Stroma Implantation for Implant Retrieval

In a separate experiment, an additional 9 mice were injected subcutaneously with 10^7 Epo^+MSCs mixed in ~500µl Contigen. At day 24 post-implantation, the organoids were removed from 4 mice anaesthetized by isoflurane inhalation as illustrated in Figure 3. The organoid was left intact in the remaining 5 "positive control" mice. Blood samples were collected prior to and every ~1week post-implantation from all 9 mice until day 77 and hematocrits assessed by standard microhematocrit method. Supplementary "negative control" mice were generated by subcutaneous administration of EmptyVector engineered MSCs embedded in collagen matrix, IRES-EGFP gene-modified MSCs admixed in collagen matrix, as well as collagen matrix only (n=3 per group). Implants were harvested from these control mice at day 23-24 ensuing implantation.

Contigen Implant Processing and Analysis

All implants recovered from mice were divided in two parts. One part of each sample was fixed with 10% formalin, embedded in paraffin and sections of 5 µm prepared and stained with hematoxylin and eosin, visualized with a microscope, and digital images saved on a computer. The other part was cut into little pieces and then treated with type IV collagenase (Sigma-Aldrich Canada Ltd, Oakville, Ontario) 1.6 mg/ml, and DNAse I (Sigma) 200 µg/ml in 1X PBS at 37°C for 1 hour. The cells that were recovered were counted and analyzed by flow cytometry for specific cell surface antigen expression as indicated earlier above.

IRES-GFP gene-modified MSCs were analyzed, as described above, prior to implantation as well as 23 days following subcutaneous injection of cells mixed in Contigen for expression of cell surface antigens exclusively on GFP positive MSCs.

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TITLES AND LEGENDS TO FIGURES

Figure 1. Long-term hematocrit of mice implanted subcutaneously with Epo-secreting marrow stroma with or without Contigen. A clonal population of Epo-gene-modified MSCs secreting 3U of Epo/ million cells/ 24 hours *in vitro* was injected, at 10^7 cells/mouse, either mixed in Contigen (full triangle) or without a matrix support (empty circle), subcutaneously in C57Bl/6 syngeneic mice (n=5 per group). Peripheral whole blood was collected from the saphenous vein for over 250 days and hematocrit measured. Control mice (full square) (n=3) were implanted with Contigen alone. Mean ± SEM.

Figure 2. Plasma Epo concentration of mice implanted with collagen-embedded Epo-secreting marrow stroma. A clonal population of Epo^+ MSCs secreting *in vitro* 3U of Epo/ million cells/ 24 hours was injected subcutaneously in syngeneic mice and saphenous vein blood was collected for over 250 days. Plasma was recovered from the peripheral blood and plasma mEpo concentration determined by ELISA specific for hEpo. Mean (n=5) ± SEM.

Figure 3. Surgical organoid implant retrieval. A group of mice injected with Epo^+MSCs-containing Contigen implants were anesthetized by isofluorane inhalation and implant removal executed as illustrated. A small skin incision was first performed, exposing the subcutaneous neo-organoid subsequently pulled out by complete and easy detachment from the host.

Figure 4. Removal of organoid implants and effect on hematocrit. Mice were injected subcutaneously with Contigen-embedded Epo^+MSCs secreting 3U of Epo/ million cells/ 24 hours. At 24 days post-implantation, the neo-organoid was excised out of several recipient mice. Hematocrit was assessed in these mice (full square)(n=4) as well as in animals with implant left intact (empty circle)(n=5).

Figure 5. Histologic analysis of collagen-based organoid implants. Contigen implants without MSCs, with Empty Vector MSCs, or with Epo^+MSCs were recovered from mice at 23-24 days post-implantation and sections stained with hematoxylin and eosin. (A) Representative section of whole implant illustrating host-derived capsule (grey arrow), Contigen band (blue arrow) and Contigen core (yellow arrow) components (4X magnification). (B) Representative section of implant capsule composed of host-derived tissues including capillary filled with red blood cells (grey arrow), neutrophils (blue arrow), and plasma cell with Russell bodies (yellow arrow) (40X magnification).

Figure 6. Phenotypic analysis of MSCs. IRES-GFP-gene-modified MSCs were analyzed by flow cytometry prior to subcutaneous implantation in mice. Expression of cell surface antigens CD31, CD34, CD44 and CD45 on GFP positive MSCs prior to implantation (Figure 6A) was evaluated as described in Materials and methods. The dashed line represents the isotype control, and the solid line represents the specific antibodies. Analysis performed 23 days following subcutaneous injection of cells *in vivo* mixed in Contigen revealed in the 3 mice similar findings for expression of CD44 and CD45 by the retrieved GFP^+MSCs (Figure 6B). There were not sufficient cells recovered from implants to allow accurate analysis of CD31 and CD34 levels.

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Figure 1

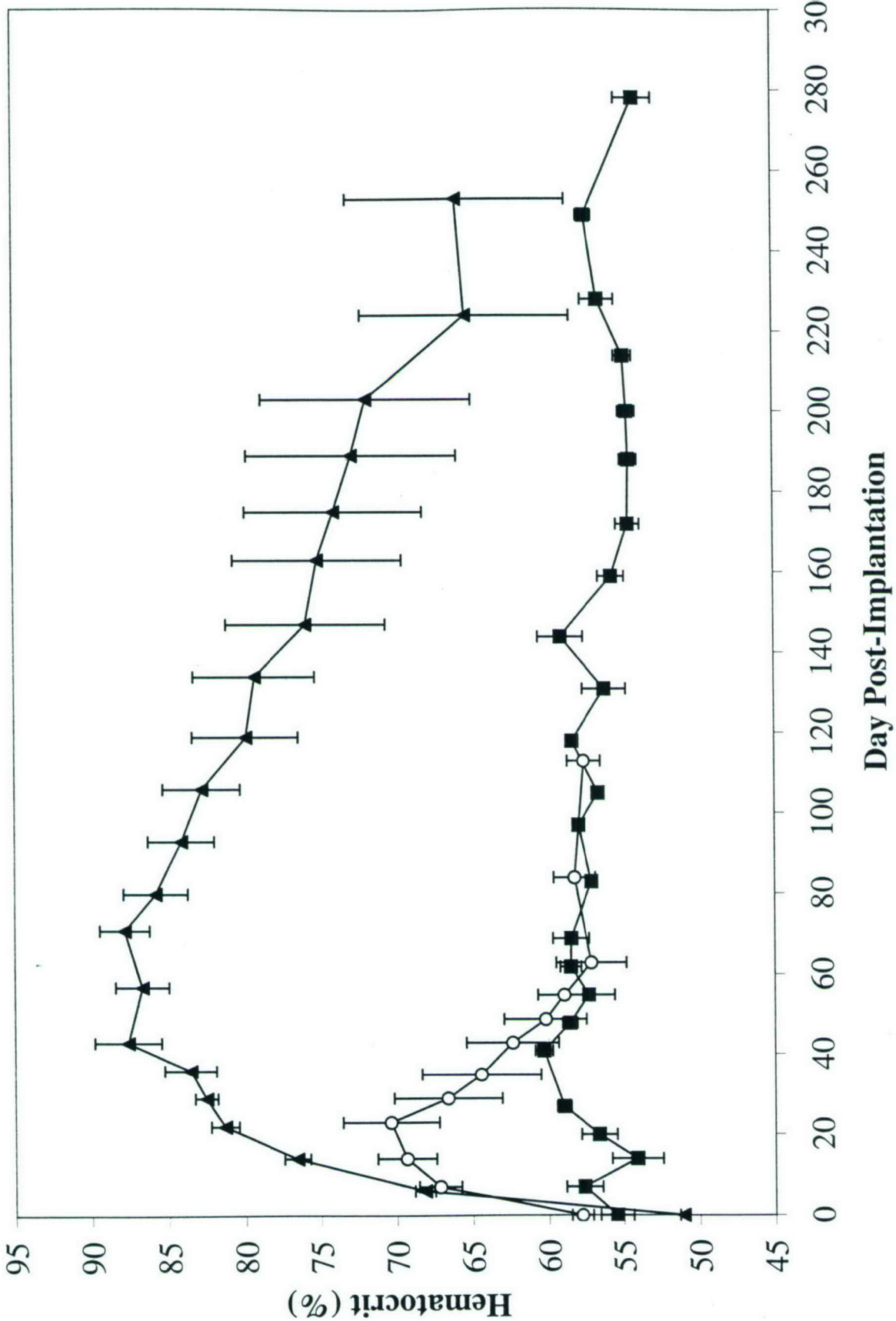

Hematocrit (%)
95
90
85
80
75
70
65
60
55
50
45
0
20
40
60
80
100
120
140
160
180
200
220
240
260
280
300
Day Post-Implantation

Figure 2

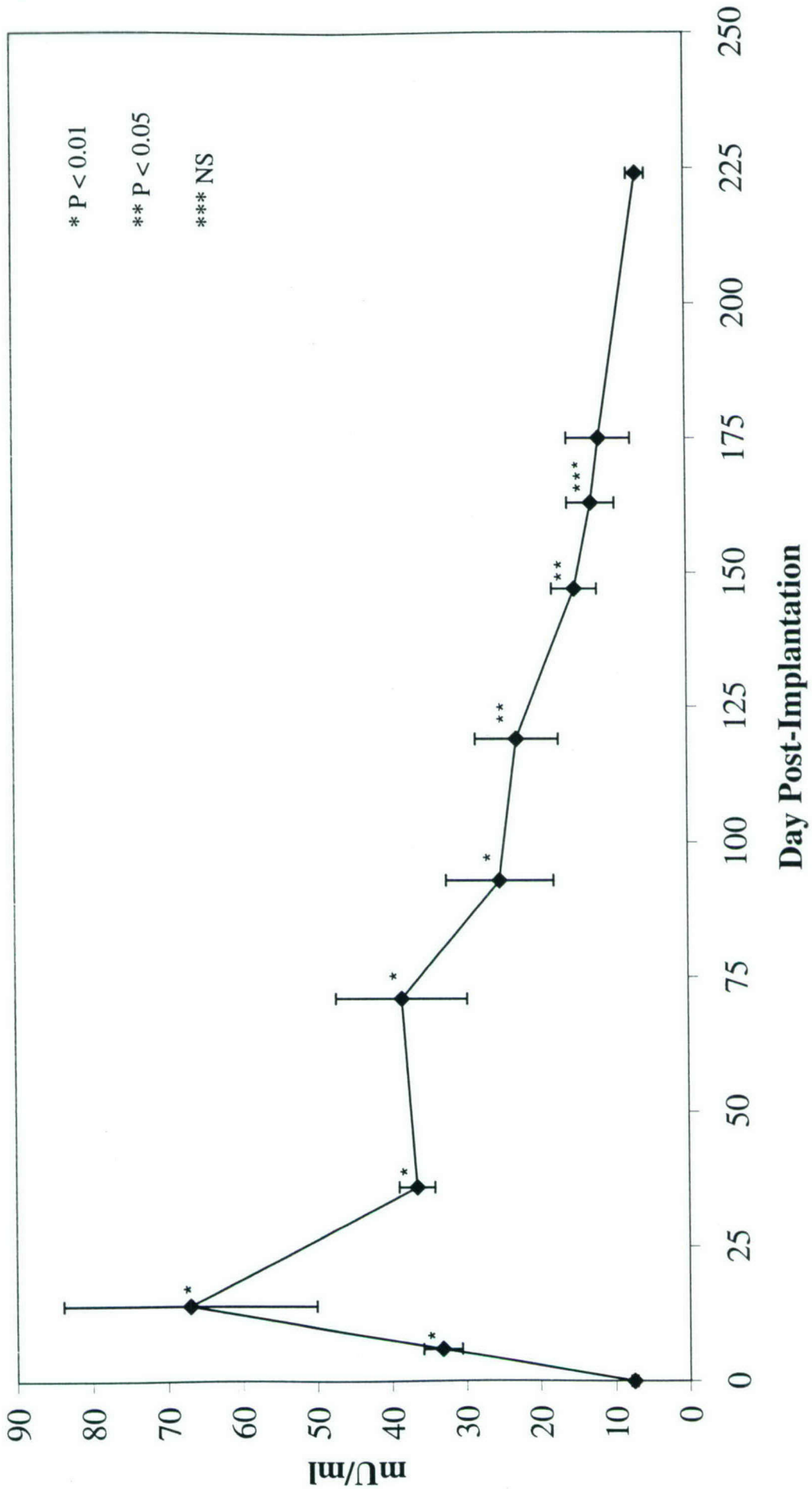
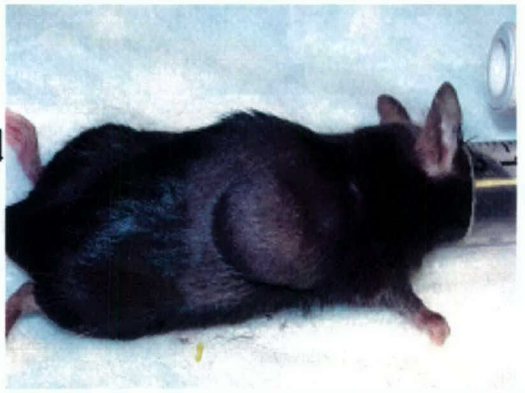
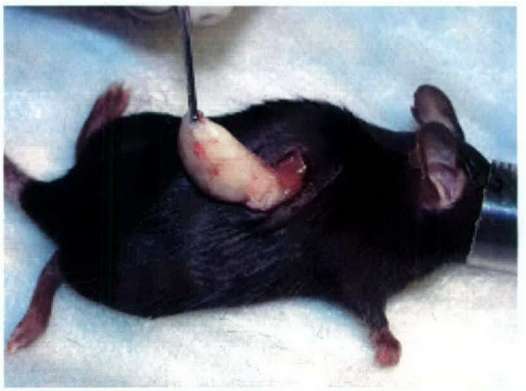

* P < 0.01
** P < 0.05
*** NS
mU/ml
0
10
20
30
40
50
60
70
80
90
0
25
50
75
100
125
150
175
200
225
250
Day Post-Implantation

Figure 3

Mouse with Contigen Implant Anesthetized by Isofluorane Inhalation for Surgery



Retrieval of Contigen Implant



Post-Removal of Contigen Implant

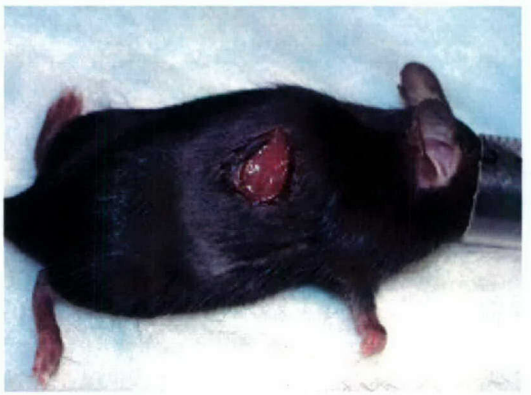


Figure 4

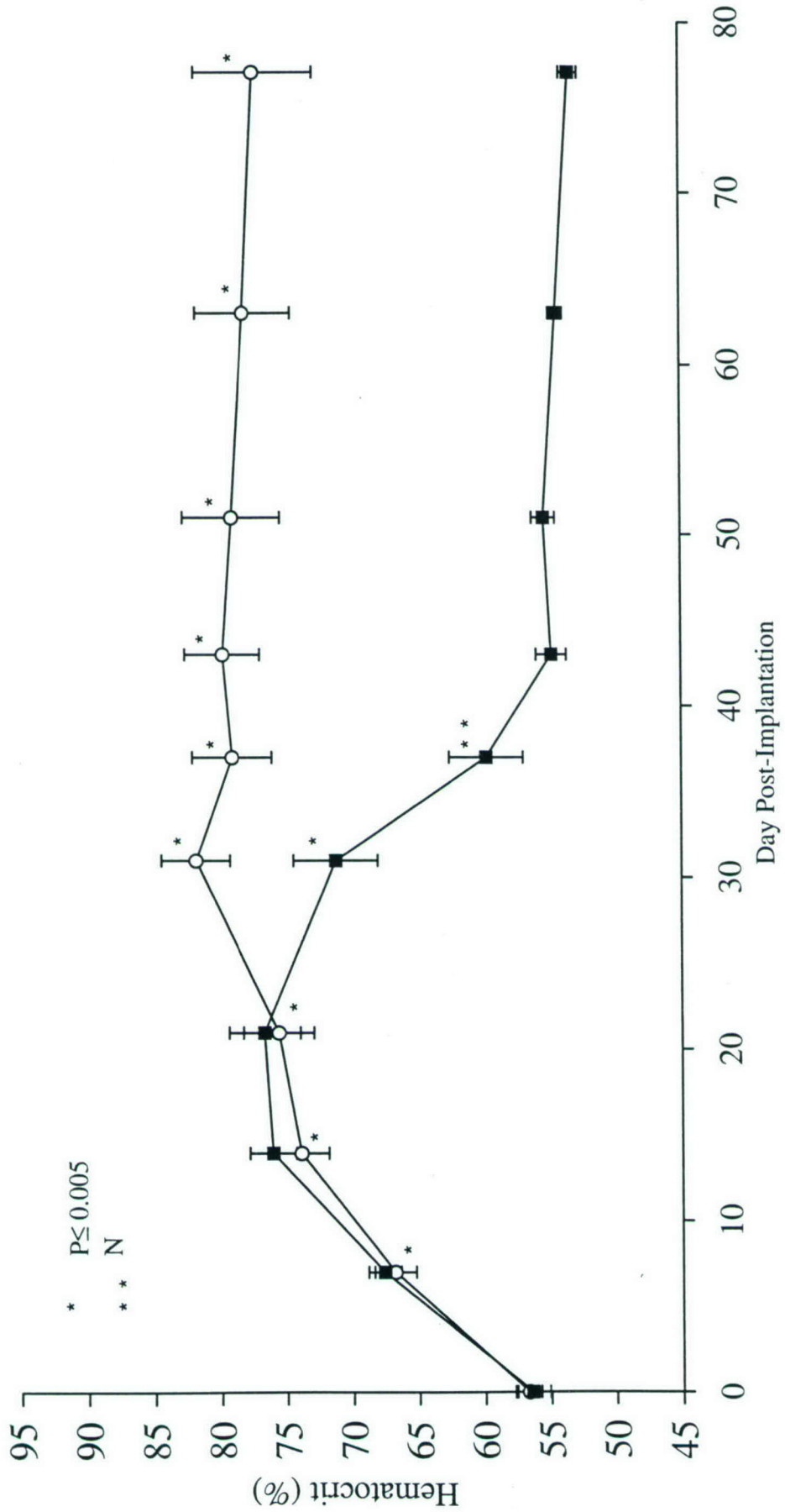
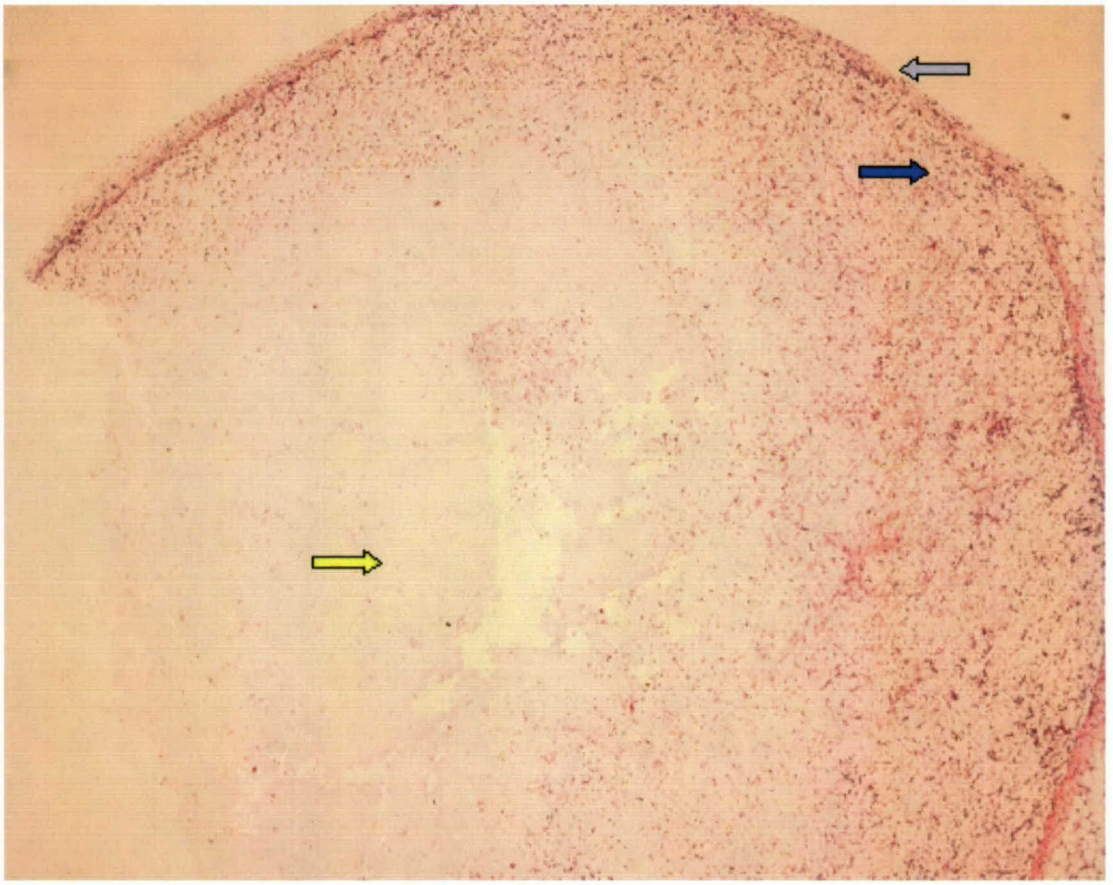

Hematocrit (%)
95
90
85
80
75
70
65
60
55
50
45
0
10
20
30
40
50
60
70
80
Day Post-Implantation
* P≤ 0.005
* * N

Figure 5

A.



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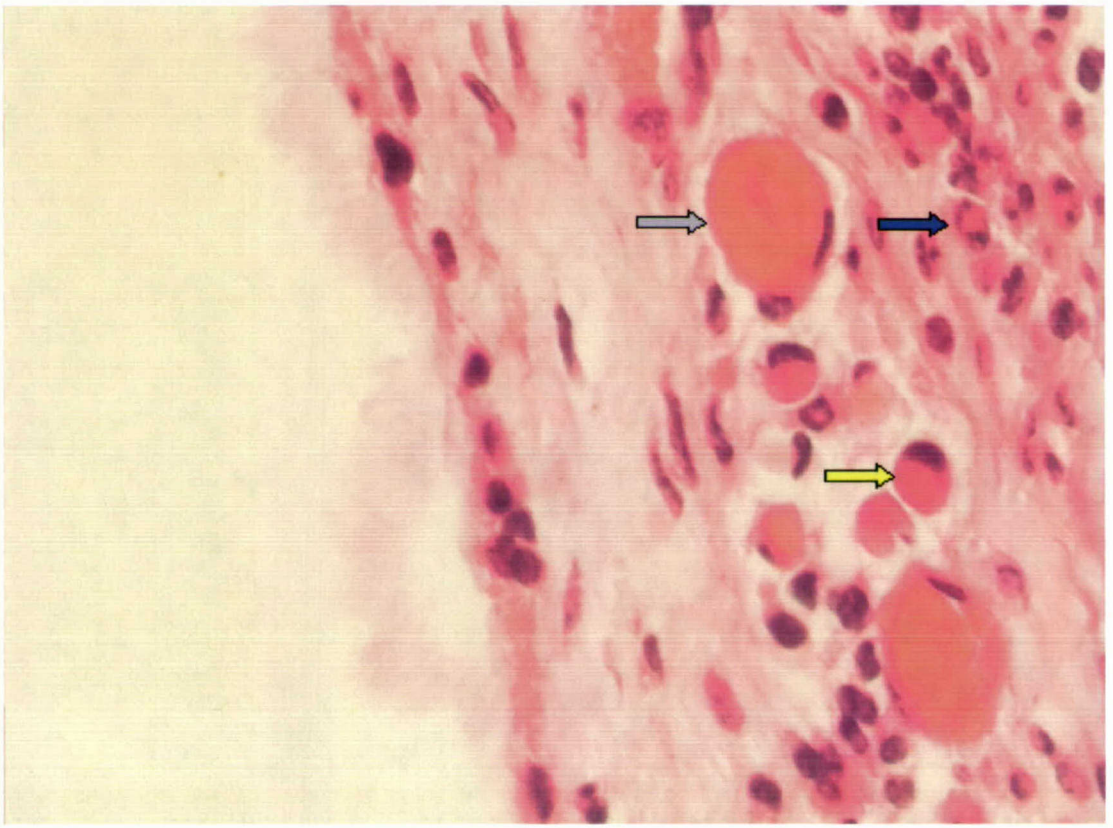
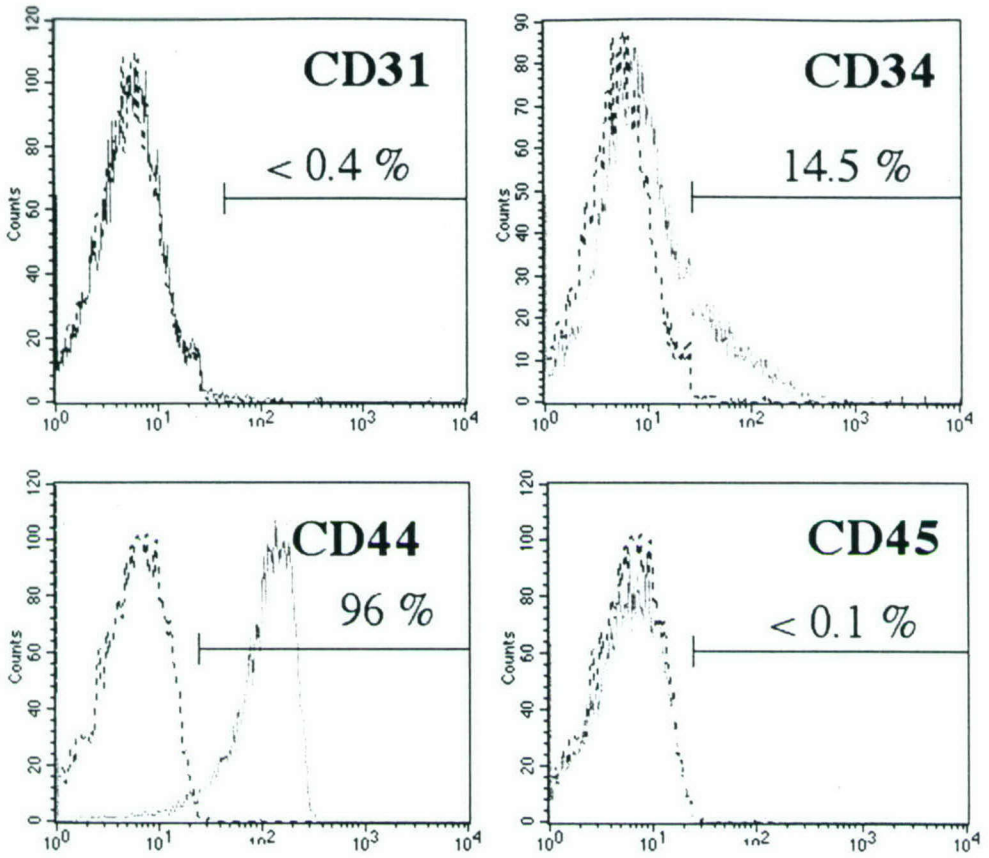


Figure 6

A



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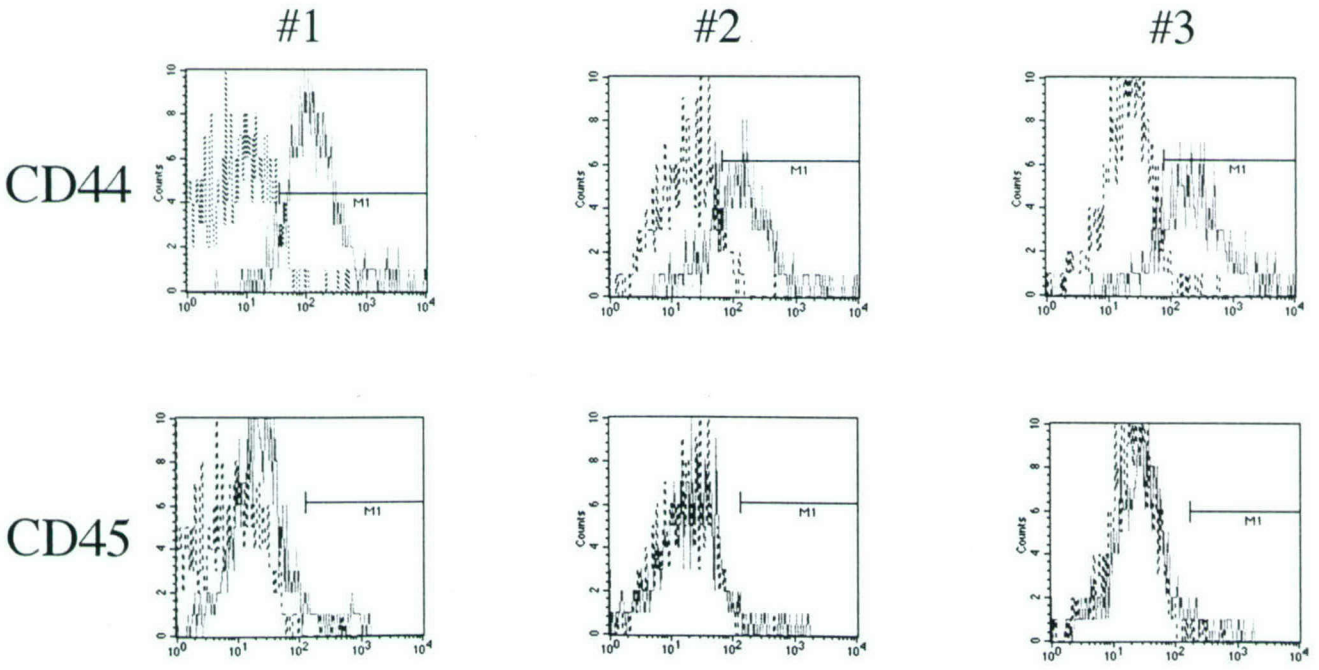

APPENDIX 3

Abstract entitled "MHC Class I and II Mismatched Marrow Stromal Cells from C57Bl/6 Mice are Immune Rejected by Recipient Balb/c Mice" by **Eliopoulos, N.**, Stagg, J., Lejeune, L., and Galipeau, J. Presented at the American Society of Gene Therapy 7th Annual Meeting, June 2-6, 2004, Minneapolis, and Figures for a manuscript in preparation.

Abstract Directory

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[911] MHC Class I and II Mismatched Marrow Stromal Cells from C57Bl/6 Mice Are Immune Rejected by Recipient Balb/c Mice

Nicoletta Eliopoulos, John Stagg, Laurence Lejeune, Jacques Galipeau Lady Davis Institute for Medical Research, McGill University, Montreal, QC, Canada; Division of Hematology/Oncology, Jewish General Hospital, McGill University, Montreal, QC, Canada

Bone marrow stromal cells (MSCs) can be easily harvested, culture expanded, and genetically engineered, and thus can be utilized therapeutically in regenerative medicine and in transgenic cell therapy strategies for delivery of therapeutic proteins. Recent studies have suggested that MSCs possess immunosuppressive properties and can be "tolerated" in allogeneic transplant setting without immunosuppression of the host. The objective of our investigation was to determine if the implantation of primary murine MSCs genetically engineered to release a soluble protein, erythropoietin (Epo), would be feasible in MHC-mismatched allogeneic mice without immunosuppression. Firstly, primary MSCs from donor C57Bl/6 (H-2K^b, H-2D^b, I-A^b) mice were retrovirally engineered to release murine Epo (Epo$^+$MSCs). These cells were admixed in a collagen-based matrix at 20 million cells/ml. Balb/c (H-2K^d, H-2D^d, I-A^d, I-E^d) mice are class I and II MHC-mismatched relative to C57Bl/6 donors and 5 recipient Balb/c mice were injected subcutaneously with 0.5 ml of Epo$^+$MSCs/matrix. In parallel, 5 recipient syngeneic C57Bl/6 mice were likewise implanted with Epo$^+$MSCs. In these latter syngeneic recipients, the hematocrit (Hct) rapidly rose from a baseline 54 ± 0.6 % (mean ± SEM) to 86 ± 0.3 % within 4 weeks post-implantation and remained >88 % for >200 days. However, in MHC-mismatched recipient Balb/c mice, the Hct rose from a basal 56 ± 0.6 % to a peak 79 ± 2.0 % at 4 weeks post-implantation and then rapidly declined to baseline values by day 52. Moreover, when these allogeneic mice received a 2nd implant of the same Epo$^+$MSCs, the Hct increase was significantly more modest and of shorter duration. A 3rd identical implant in these same Balb/c mice led to no significant effect on Hct. Repeat experiments performed in the absence of collagen matrix led to similar results. To elucidate the host-derived immune response to the MHC-mismatched Epo$^+$MSC implant, allogeneic Balb/c and syngeneic C57Bl/6 mice were identically implanted with C57Bl/6 derived Epo$^+$MSCs, sacrificed at day 15 and implants recovered. Implants were collagenase dissociated and flow cytometric analysis conducted on host-derived lymphoid cells revealed that allogeneic implants compared to syngeneic comprised significantly greater proportions of CD8+, NK T, CD11c, and NK cells. These results are consistent with a robust host cellular immune response to donor allogeneic Epo$^+$MSCs. To determine whether Epo production was causative in the observed immune response, "null" C57Bl/6 MSCs, lacking Epo production, were implanted in Balb/c mice and splenocytes from recipients were isolated 15 days later and tested for their interferon gamma (IFNγ) activation by C57Bl/6 MSCs *in vitro*. We found that splenocytes from these test Balb/c mice displayed a robust, specific, and significant IFNγ response to C57Bl/6 MSCs when compared to controls. Therefore, our results reveal that class I and II MHC-mismatched MSCs will elicit a cellular immune response by allogeneic hosts with normal immune systems, and that rejection is amplified by repeated challenge. These results strongly suggest that MSCs, at least in the murine system, cannot serve as a "universal donor" in cell therapy applications.

Keywords: Stem Cell - mesenchymal; New Technologies; Viral Gene Transfer

Saturday, June 5, 2004 4:00 PM

Poster Session III: Gene Transfer Applications for Disorders of Hematopoietic Cells (4:00 PM-7:00 PM)

Close Window

Figure 1

Experimental Outline of Implantations

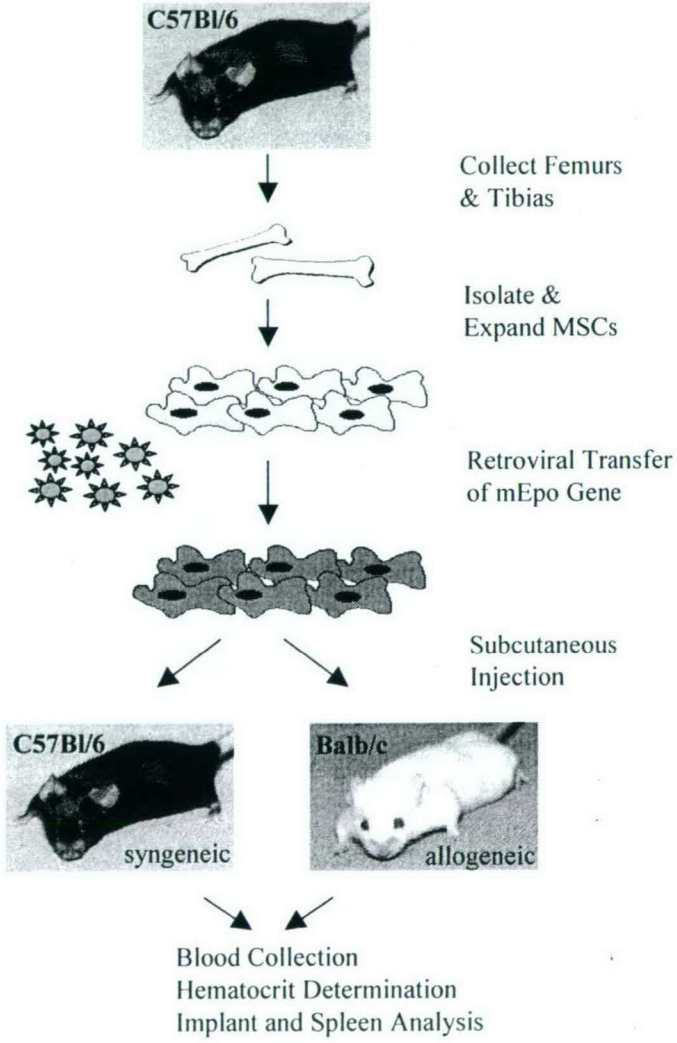


Figure 2

Phenotypic Analysis of Epo^{+} MSCs

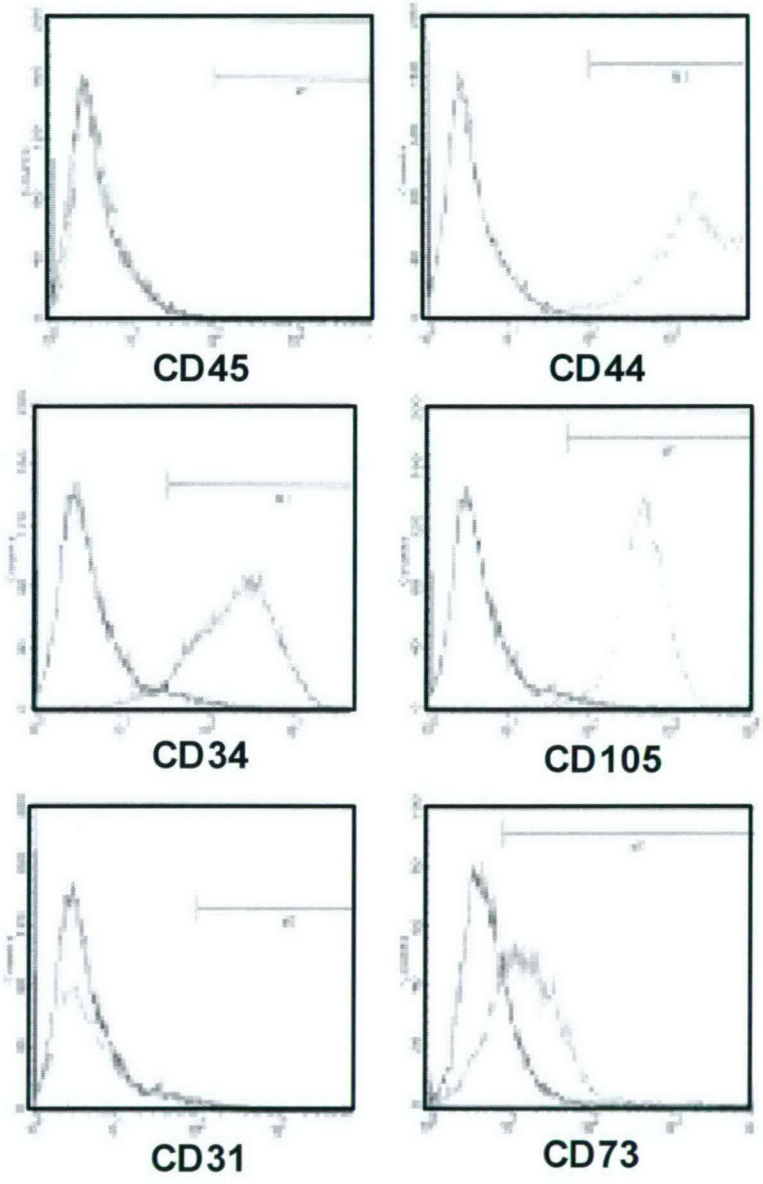


Figure 3

Hematocrit of C57Bl/6 and Balb/c Mice Implanted with Epo-Secreting C57Bl/6-Derived MSCs

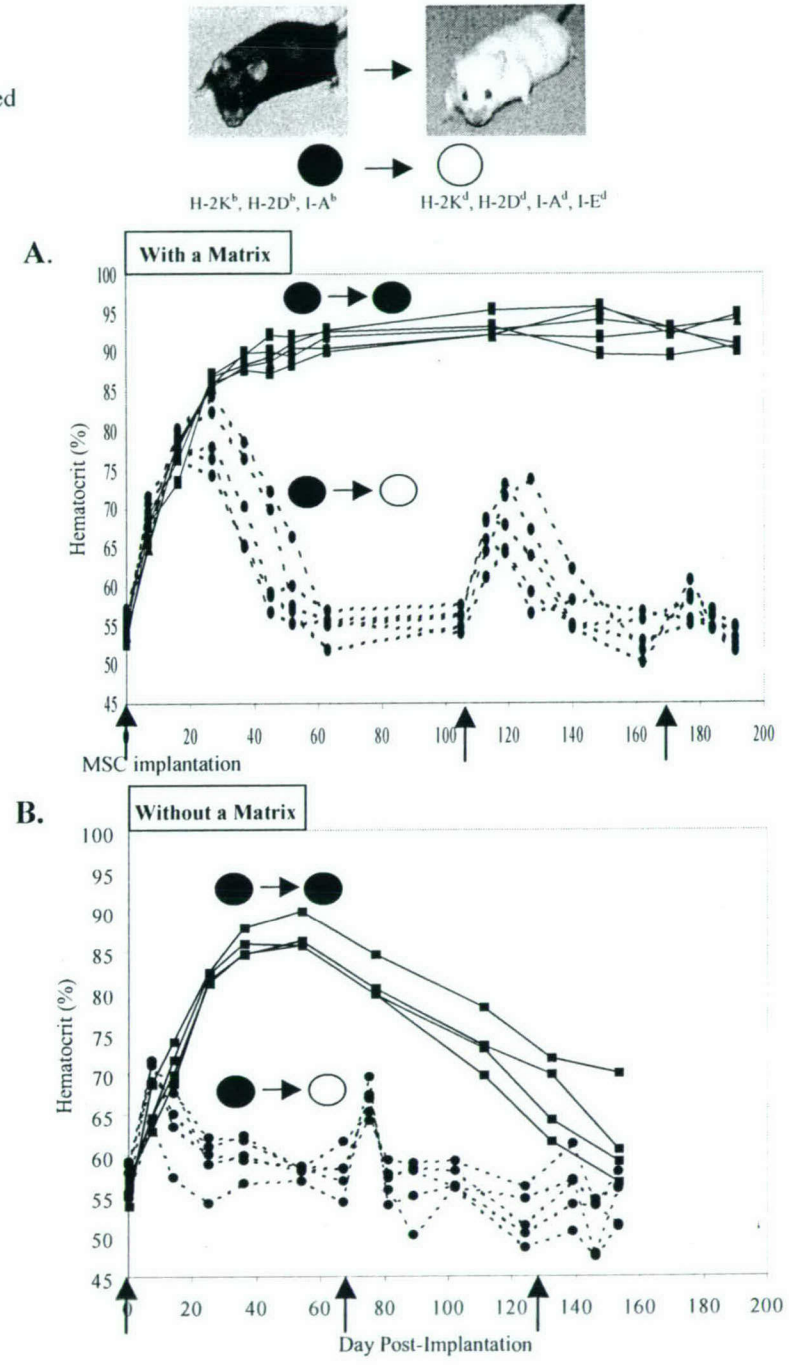


Figure 4

Analysis of Cells Isolated from Retrieved Implants

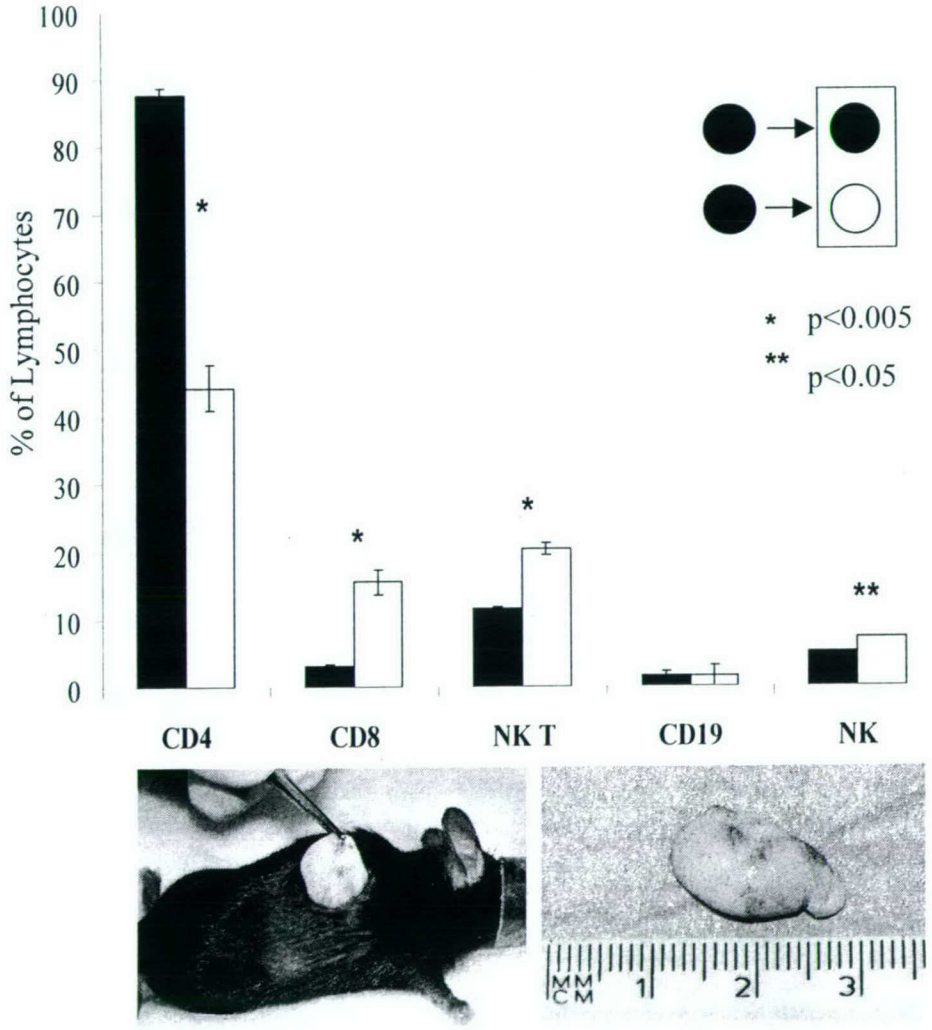


Figure 5

IFN-γ Release by Retrieved Splenocytes

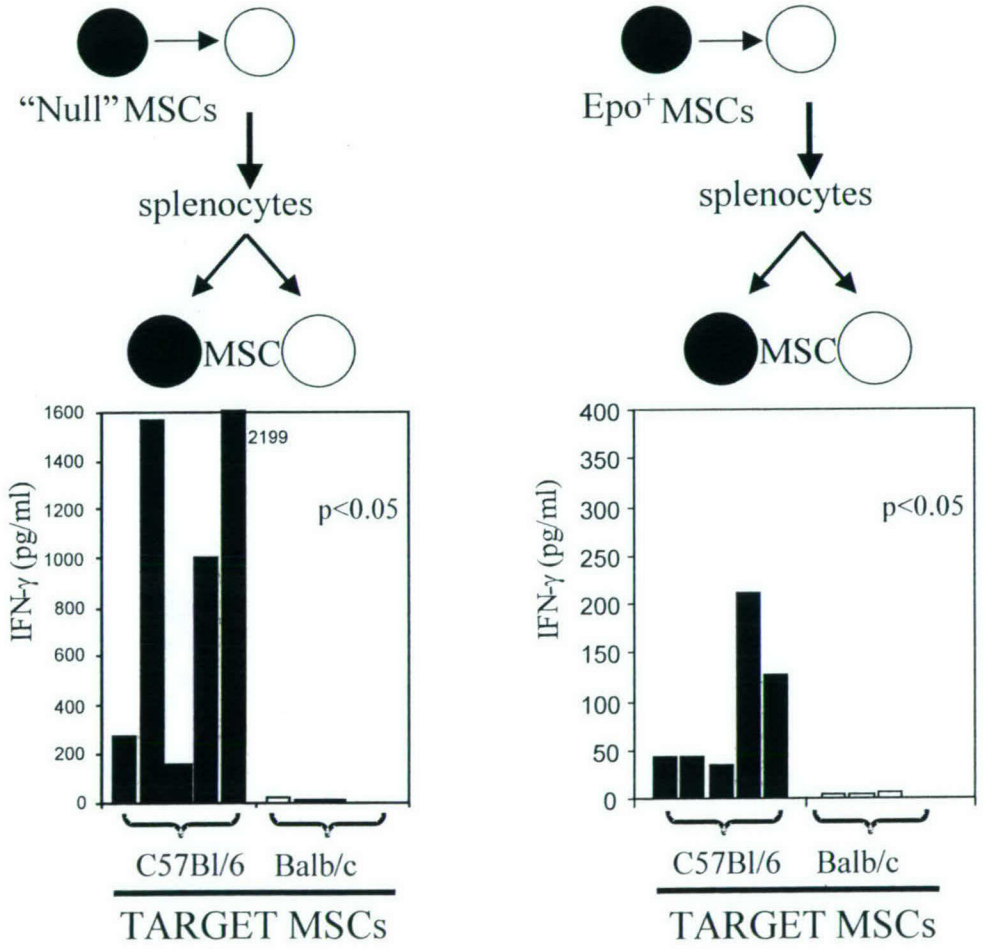
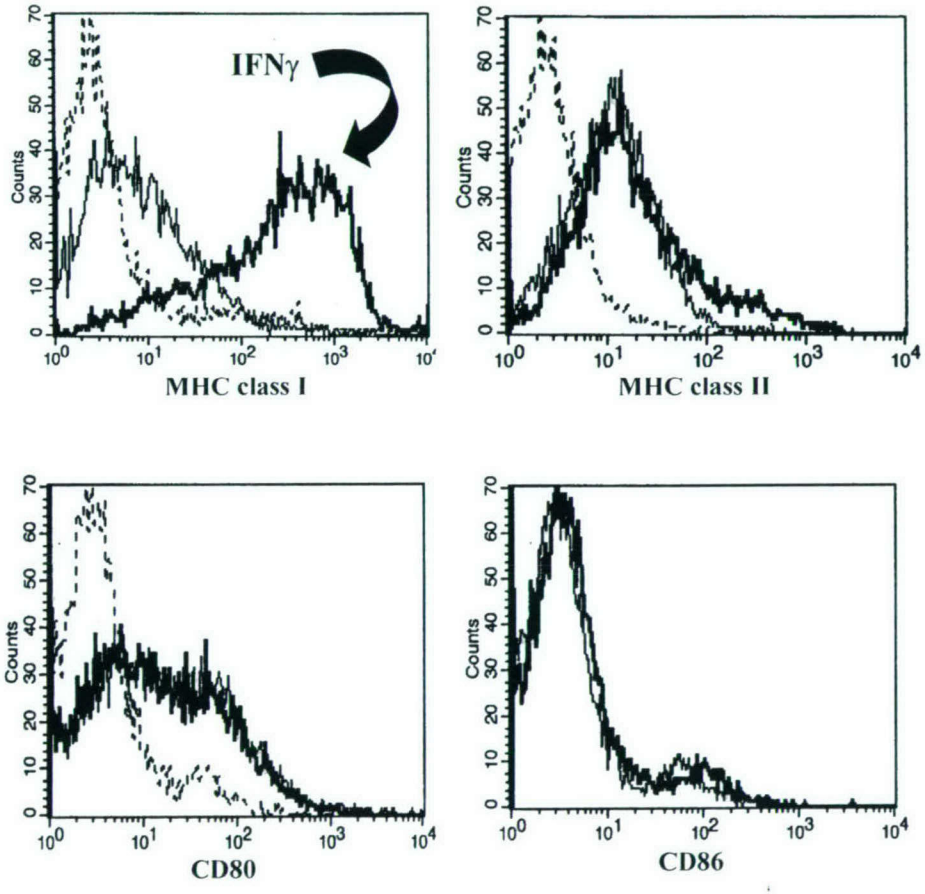


Figure 6

Analysis of Epo$^+$ MSCs Pre- and Post-IFN-γ Exposure



APPENDIX 4

Certificate obtained through participation in the course entitled "Critical Issues in Tumor Microcirculation, Angiogenesis and Metastasis: Biological Significance and Clinical Relevance" offered by Harvard Medical School, Department of Continuing Education, and Massachusetts General Hospital, in June 2004, in Cambridge, MA.

HARVARD MEDICAL SCHOOL

DEPARTMENT OF CONTINUING EDUCATION

BOSTON, MASSACHUSETTS

CERTIFIES THAT

Nicoletta Eliopoulos

has participated in the educational activity titled

Critical Issues in Tumor Microcirculation, Angiogenesis and Metastasis

June 7-10, 2004

The activity was designated for 24.00 category 1 credits toward the AMA/PRA.

Faculty Dean for Continuing Education
Sanjiv Chopra, MB,BS

LECTURE SCHEDULE

Critical Issues In Tumor Microcirculation, Angiogenesis and Metastastis:

Biological Significance and Clinical Relevance

June 7 – 10, 2004

Monday, June 7, 2004

7:30 A.M.	*Registration*	
8:00 A.M.	*Continental Breakfast*	
8:30 A.M.	**Opening Remarks**	Jain
9:00 A.M.	**Tumor Angiogenesis - I:** **The Genetic Basis of the Angiogenic Switch**	Folkman
10:00 A.M.	*Coffee Break*	
10:30 A.M.	**Tumor Angiogenesis - II:** **Clinical Applications of Angiogenesis Research**	Folkman
Noon	*Lunch*	
1:30 P.M.	**Tumor Angiogenesis - III:** **Future Directions: Can the Angiogenic Switch be Prevented?**	Folkman
3:00 P.M.	*Coffee Break*	
3:30 P.M.	**Combating Drug Resistance and Toxicity with Metronomic Chemotherapy and Targeted Antiangiogenics**	Kerbel
5:00 P.M.	*Adjourn*	

Wednesday, June 9, 2004

8:00 A.M.	*Continental Breakfast*	
8:30 A.M.	**Delivery of Molecular Medicine to Tumors - III: Interstitial and Lymphatic Transport**	Jain
10:00 A.M.	*Coffee Break*	
10:30 A.M.	**Delivery of Molecular Medicine to Tumors - IV: Cell-Based Therapeutics**	Jain
Noon	*Lunch Break (on your own)*	
1:30 P.M.	**Role of Adhesion Molecules in Tumor Growth, Angiogenesis and Metastasis - I: General Principles**	Hynes
3:00 P.M.	*Coffee Break*	
3:30 P.M.	**Role of Adhesion Molecules in Tumor Growth, Angiogenesis and Metastasis - II: Integrins**	Hynes
5:00 P.M.	*Adjourn*	

Harvard Medical School

Department of Continuing Education

Critical Issues in Tumor Microcirculation, Angiogenesis and Metastasis

June 7-10, 2004

Dr. Adam W. Beck	*Dallas, TX*
Mr. Stephen P. Bradley	*Cambridge, MA*
Mr. Dave N. Cervi	*Toronto, ON*
Dr. Soo-Ik Chang	*Seoul, Korea*
Dr. Shuk Han Cheng	*Hong Kong, Hong Kong*
Ms. Claudia Chiodoni	*Milan, Italy*
Mr. John Edwards	*Waltham, MA*
Dr. Nicoletta Eliopoulos	*Montreal, Quebec, Canada*
Ms. Mira Ernkvist	*Stockholm, Sweden*
Dr. Janos Geli	*Stockholm, Sweden*
Dr. Dilara Grate	*Cambridge, MA*
Dr. Alison M. Griffen	*Cheshire, United Kingdom*
Dr. Alan S. Harris	*Waltham, MA*
Ms. Anja M. Hoeg	*Copenhagen, Denmark*
Mr. Magnus Johansson	*Uppsala, Sweden*
Dr. Linda Kalikin	*Ann Arbor, MI*
Dr. Amin I. Kassis	*Boston, MA*
Dr. Joji Kitayama	*Tokyo, Japan*
Dr. Grzegorz Korpanty	*Dallas, TX*
Ms. Mona Larsen	*Copenhagen, Denmark*
Ms. Joey Lau	*Uppsala, Sweden*
Dr. Intae Lee	*Philadelphia, PA*
Mr. Carsten D. Ley	*Copenhagen, Denmark*
Dr. Robert Loberg	*Ann Arbor, MI*
Dr. Sharon R. Lubkin	*Raleigh, NC*
Dr. Gary Mac Vicar	*Ann Arbor, MI*
Dr. Al Malkinson	*Denver, CO*
Dr. Georgia Mavria	*London, United Kingdom*
Dr. Thomas G. McCauley	*Cambridge, MA*
Dr. Takeshi Morii	*Tokyo, Japan*
Dr. Hideo Morioka	*Tokyo, Japan*
Dr. Sabeeha Muneer	*Dallas, TX*
Dr. Vasanti Natarajan	*Oslo, Norway*
Mr. Chris Neeley	*Ann Arbor, MI*
Dr. Fiemu Nwariaku	*Dallas, TX*
Dr. Thomas P. O'Connor	*West Seneca, NY*
Mr. Anthony O'Grady	*Dublin, Ireland*
Dr. Richard F. Olsson	*Uppsala, Sweden*
Dr. Lone H. Ottesen	*Copenhagen, Denmark*
Dr. Massimo Pinzani	*Firenze, Italy*
Dr. Yi Wei Qi	*Cambridge, MA*
Mr. Christian R. Schnell	*Basle, Switzerland*
Mr. Taro Semba	*Tsukuba, Japan*
Dr. Mukund Seshadri	*Buffalo, NY*
Dr. Lily S. Shahied	*Radnor, PA*
Dr. Sreesha Srinivasa	*Branford, CT*
Dr. Cliona M. Stapleton	*Research Triangle Park, NC*
Dr. Paul C. Stomper	*Buffalo, NY*
Dr. Katsuhito Takeuchi	*Tokyo, Japan*
Dr. Koichiro Tanaka	*Tokyo, Japan*
Dr. Kwanchanit Tantivejkul	*Ann Arbor, MI*
Dr. Lyann May B. Ursos	*Willowbrook, IL*
Ms. Britta Weigelt	*Amsterdam, The Netherlands*
Dr. Joseph T. Wu	*Atlanta, GA*
Dr. Dahai Xue	*West Haven, CT*
Dr. Hiroharu Yamashita	*Tokyo, Japan*
Mr. Steffen M. Zeisberger	*Villigen-PSI, Switzerland*